



11 October 2013 | \$10

Science

EDITORIAL

- 161 Shutdown!
Marcia McNutt

NEWS OF THE WEEK

- 172 A roundup of the week's top stories

NEWS & ANALYSIS

- 175 Higgs and Cell Studies Nab Nobels
176 U.S. Shutdown Tightens Its Grip on Research
177 NASA Meeting Bars Chinese Students
178 Dutch H5N1 Ruling Raises New Questions
179 Severe Autism, Often Slighted, Now Targeted for Study
181 Farming's Tangled European Roots
 >> Report p. 257; Science Express Report by R. Bollongino et al.
182 Windfall for Tiny University With Outsized Ambitions

NEWS FOCUS

- 183 Impact Theory Gets Whacked
 >> Science Podcast
186 Biology's Dry Future

LETTERS

- 191 Retraction
S.-W. Lee et al.
Working Together to Prepare for Disasters
K. L. Rising and N. Lurie
A Model of Strength
D. H. Johnson and R. D. Cook
The Editor's Dilemma
D. E. Chubin
193 CORRECTIONS AND CLARIFICATIONS

BOOKS ET AL.

- 195 *Artemisia annua*, Artemisinin, ACTs and Malaria Control in Africa
D. G. Dalrymple, reviewed by W. Lawley et al.
196 Irrationality in Health Care
D. E. Hough, reviewed by R. S. Mathis

POLICY FORUM

- 197 Newborn Screening: Gaps in the Evidence
B. Wilcken
 >> Science Podcast

PERSPECTIVES

- 200 What Are Mini-Brains?
B. Bae and C. A. Walsh
201 Structure and Motion of a 2D Glass
M. Heyde
 >> Report p. 224
202 Directing Data Center Traffic
Y. Fainman and G. Porter
203 Getting Your Gut into Shape
B. D. Simons
 >> Research Article p. 212
205 Cleaner Lakes Are Dirtier Lakes
E. S. Bernhardt
 >> Report p. 247
206 GWAS to Therapy by Genome Edits?
R. C. Hardison and G. A. Blobel
 >> Report p. 253
207 RNAi, Antiviral After All
S. M. Sagan and P. Sarnow
 >> Reports pp. 231 and 235

CONTENTS continued >>



page 183



page 200

ON THE WEB THIS WEEK

- >> **Science Podcast**
Listen to stories about gaps in the evidence for newborn screenings, the formation of the Moon, removing nitrogen from lakes, and more.
>> **Find More Online**
Check out Science Express, our podcast, videos, daily news, our research journals, and Science Careers at www.sciencemag.org.



COVER

False-colored scanning electron micrograph of erythrocytes (diameter ~7 micrometers). Sickle cell disease results from an inherited defect of adult hemoglobin, the oxygen-carrying metalloprotein constituent of erythrocytes. Common genetic variation associated with sickle cell disease severity modulates an adult-stage erythroid enhancer element of the *BCL11A* gene. Disruption of this element could ameliorate the disease by reestablishing expression of fetal hemoglobin. See pages 206 and 253.

Image: Dennis Kunkel Microscopy, Inc./Visuals Unlimited, Inc.

DEPARTMENTS

- 159 This Week in Science
163 Editors' Choice
170 Science Staff
265 New Products
266 Science Careers

REVIEW

- 210 **Fueling Immunity: Insights into Metabolism and Lymphocyte Function**
E. L. Pearce et al.
Review Summary; for full text:
<http://dx.doi.org/10.1126/science.1242454>

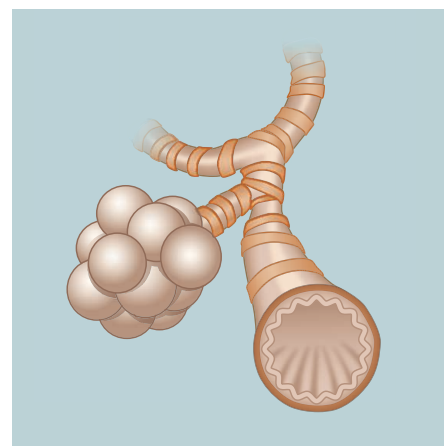
RESEARCH ARTICLES

- 211 **Opposite Feedbacks in the Hippo Pathway for Growth Control and Neural Fate**
D. Jukam et al.
Hippo directs cell differentiation and fate through context- and tissue-specific feedback and transcription networks.
Research Article Summary; for full text:
<http://dx.doi.org/10.1126/science.1238016>
- 212 **Villification: How the Gut Gets Its Villi**
A. E. Shyer et al.
Muscular control over proliferating mesenchyme and epithelium yields intestinal villi.
>> Perspective p. 203

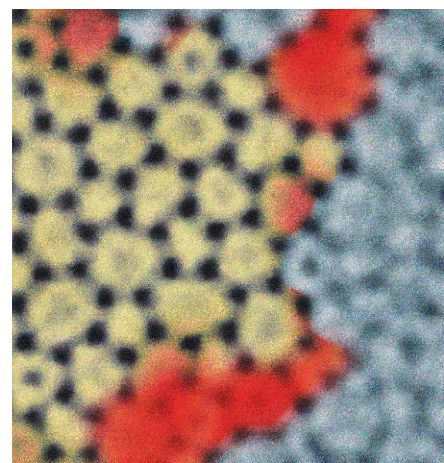
REPORTS

- 218 **Evidence for Water in the Rocky Debris of a Disrupted Extrasolar Minor Planet**
J. Farihi et al.
Spectroscopic analysis of a debris-accreting star in its latest stage of life reveals the remnants of a water-bearing world.
- 220 **Femtosecond Visualization of Lattice Dynamics in Shock-Compressed Matter**
D. Milathianaki et al.
The response to shock in polycrystalline copper is seen to evolve from elastic to plastic using ultrafast x-ray diffraction.
- 224 **Imaging Atomic Rearrangements in Two-Dimensional Silica Glass: Watching Silica's Dance**
P. Y. Huang et al.
Dynamics of individual atoms in a two-dimensional silicate glass have been observed using transmission electron microscopy.
>> Perspective p. 201
- 227 **Waveform Tomography Reveals Channeled Flow at the Base of the Oceanic Asthenosphere**
S. French et al.
Mantle convection produces low-wavelength fingerlike structures parallel to the directions of plate motion.

- 231 **RNA Interference Functions as an Antiviral Immunity Mechanism in Mammals**
Y. Li et al.
- 235 **Antiviral RNA Interference in Mammalian Cells**
P. V. Maillard et al.
Certain mammalian cells can use RNA interference in the innate defense against invading viruses.
>> Perspective p. 207
- 239 **RTEL1 Is a Replisome-Associated Helicase That Promotes Telomere and Genome-Wide Replication**
J.-B. Vannier et al.
A DNA helicase that protects telomeres is also required for replication of the rest of the genome.
- 243 **Structures and Receptor Binding of Hemagglutinins from Human-Infecting H7N9 Influenza Viruses**
Y. Shi et al.
Four amino acids in the H7N9 influenza virus binding site provide a hydrophobic environment for human receptors.
- 247 **Human Influences on Nitrogen Removal in Lakes**
J. C. Finlay et al.
Successful control of phosphorus levels results in nitrogen accumulation in many of Earth's largest lakes.
>> Perspective p. 205; Science Podcast
- 250 **Type 6 Secretion System-Mediated Immunity to Type 4 Secretion System-Mediated Gene Transfer**
B. T. Ho et al.
A general bacterial defense mechanism suppresses the movement of horizontally transferred DNA in bacterial populations.
- 253 **An Erythroid Enhancer of *BCL11A* Subject to Genetic Variation Determines Fetal Hemoglobin Level**
D. E. Bauer et al.
Fine-mapping reveals a promising therapeutic target for genome engineering in the β -hemoglobinopathies.
>> Perspective p. 206
- 257 **Ancient DNA Reveals Key Stages in the Formation of Central European Mitochondrial Genetic Diversity**
G. Brandt et al.
Mitochondrial DNA profiles of 364 prehistoric people reveal human demography and migration patterns in Neolithic Germany.
>> News story p. 181



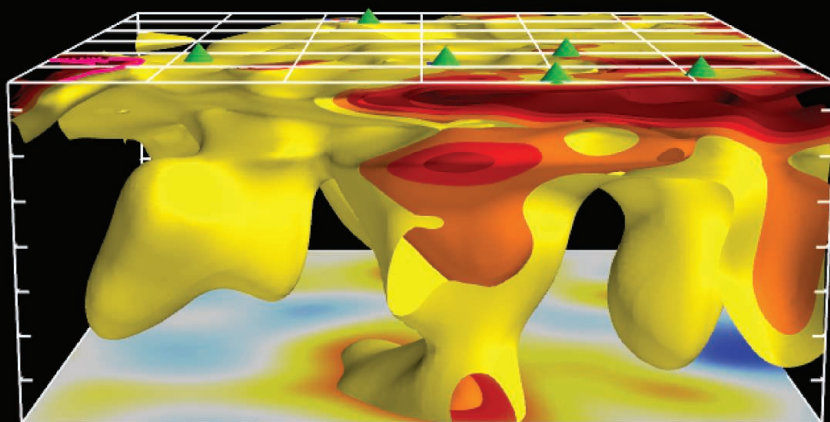
pages 203 & 212



pages 201 & 224

SCIENCE (ISSN 0036-8075) is published weekly on Friday, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue, NW, Washington, DC 20005. Periodicals Mail postage (publication No. 484460) paid at Washington, DC, and additional mailing offices. Copyright © 2013 by the American Association for the Advancement of Science. The title SCIENCE is a registered trademark of the AAAS. Domestic individual membership and subscription (51 issues): \$149 (\$74 allocated to subscription). Domestic institutional subscription (51 issues): \$990; Foreign postage extra: Mexico, Caribbean (surface mail) \$55; other countries (air assist delivery) \$85. First class, airmail, student, and emeritus rates on request. Canadian rates with GST available upon request, GST #1254 88122. Publications Mail Agreement Number 1069624. Printed in the U.S.A.

Change of address: Allow 4 weeks, giving old and new addresses and 8-digit account number. Postmaster: Send change of address to AAAS, P.O. Box 96178, Washington, DC 20090-6178. Single-copy sales: \$10.00 current issue, \$15.00 back issue prepaid includes surface postage; bulk rates on request. Authorization to photocopy material for internal or personal use under circumstances not falling within the fair use provisions of the Copyright Act is granted by AAAS to libraries and other users registered with the Copyright Clearance Center (CCC) Transactional Reporting Service, provided that \$30.00 per article is paid directly to CCC, 222 Rosewood Drive, Danvers, MA 01923. The identification code for Science is 0036-8075. Science is indexed in the Reader's Guide to Periodical Literature and in several specialized indexes.



Mapping Mantle Mixing

Mantle convection is the primary driving force for plate tectonics, but mantle convection also mixes material in the interior of Earth and controls heat flow from the core. The patterns of convection are often difficult to image directly with seismic waves—particularly on a global scale. **French *et al.*** (p. 227, published online 5 September) constructed a global tomographic model of the upper mantle and transition zone that is sensitive to changes in seismic velocity and anisotropy. The approach identifies elongated, horizontal structures in the upper mantle that are parallel to overlying plate motions. At greater depths, however, vertical plume-like structures extend from the lower mantle and disappear near the base of low velocity zones like those observed beneath Hawaii.

Intestinal Villus Formation

The intestinal villi are essential elaborations of the lining of the gut that increase the epithelial surface area for nutrient absorption. **Shyer *et al.*** (p. 212, published online 29 August; see the Perspective by **Simons**) show that in both the developing human and chick gut, the villi are formed in a step-wise progression, involving the sequential folding of the endoderm into longitudinal ridges, via a zigzag pattern, to finally form individual villi. These changes are established through the differentiation of the smooth muscle layers of the gut, restricting the expansion of the adjacent proliferating and growing endoderm and mesenchyme, generating compressive stresses that lead to the buckling and folding of the tissue.

The Origins of Europeans

To investigate the genetic origins of modern Europeans, **Brandt *et al.*** (p. 257) examined ancient mitochondrial DNA (mtDNA) and were able to identify genetic differences in 364 Central Europeans spanning the early Neolithic to the Early Bronze Age. Observed changes in mitochondrial haplotypes corresponded with hypothesized human migration across Eurasia and revealed the complexity of the demographic changes and evidence of a Late Neolithic origin for the European mtDNA gene pool. This transect through time reveals four key population events associated

with well-known archaeological cultures, which involved genetic influx into Central Europe from various directions at various times.

Elastic to Plastic

When a crystal is mechanically compressed, it first reacts elastically (reversibly), and then enters the plastic regime, in which the structure of the material is irreversibly changed. This process can be studied with molecular dynamics (MD) simulations on very fine temporal and spatial scales, but experimental analysis has lagged behind. **Milathianaki *et al.*** (p. 220) shocked polycrystalline copper with a laser beam, and then took successive snapshots of the crystal structure at 10-picosecond intervals. The results were compared directly with atomistic simulations and revealed that the yield stress—the point of transition from plastic to elastic response—agreed well with MD predictions.

Glassy Eyed

In crystalline materials, the collective motion of atoms in one- and two-dimensional defects—like dislocations and stacking faults—controls the response to an applied strain, but how glassy materials change their structure in response to strain is much less clear. **Huang *et al.*** (p. 224; see the Perspective by **Heyde**) used advanced-transmission electron microscopy to investigate the structural rearrangements in a

two-dimensional glass, including the basis for shear deformations and the atomic behavior at the glass/liquid interface.

Remnants of a Water-Bearing World

Stars like the Sun end their lives as white dwarfs. **Farihi *et al.*** (p. 218) used detailed spectroscopic analysis of a debris-accreting white dwarf, along with knowledge that such systems accrete this debris from remnants of rocky planetary bodies, to derive the water content in a disrupted extra-solar body. The findings suggest that the white dwarf contains the signature of a rocky minor planet composed of 26% water by mass.

Viral Defenses

In plants and invertebrates, RNA interference (RNAi) functions as an innate antiviral defense mechanism. Viruses that infect plants and invertebrates have evolved viral suppressors of RNAi (VSRs) that disable the RNAi pathway. Whether mammals use RNAi as a defense against viruses has been less clear (see the

Perspective by **Sagan and Sarnow**).

Li *et al.* (p. 231) and **Mail-lard *et al.*** (p. 235) studied mammalian cell lines and baby mice productively infected with RNA viruses and observed the production of virus-derived small RNAs (vsRNAs). When the putative VSR proteins of the infecting viruses were disabled, host RNAi-derived vsRNAs were much increased and the viruses were rapidly cleared and unable to mount a full-blown infection. Thus, RNAi also has an innate antiviral function in mammals.



Unlucky Lakes

The negative consequences of increased loading of nitrogen and phosphorus into aquatic ecosystems are well known. Management strategies aimed at reducing the sources of these excess nutrients, such as fertilizer runoff or sewage outflows, can largely mitigate the increases in nitrogen and phosphorus levels; however, it is unclear if these strategies are influencing other aspects of these ecosystems. Using a global lake data set, **Finlay *et al.*** (p. 247; see the Perspective by **Bernhardt**) found that reducing phosphorus inputs reduced a lake's ability to export reactive nitrogen, exacerbating nitrate pollution.

Additional summaries

Lymphocyte Metabolism

Lymphocytes are highly dynamic cells, undergoing extensive proliferation upon infection and then reducing in number upon pathogen clearance. Lymphocytes also circulate through many different tissue environments that vary in their nutrient and oxygen availability. Recent studies have revealed changes in metabolic programming that facilitate this dynamic behavior. How these changes occur and the specific effects that they have on lymphocyte function and on the ultimate outcome of an infection are not well understood. **Pearce *et al.*** (p. 210) review recent progress in this area, suggest how parallels might be found in studying the metabolic changes seen in tumor cells, and propose challenges for the future.

Complexity and Diversity

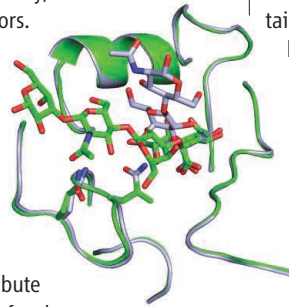
Complex organisms must produce and maintain an extraordinary diversity of cell and tissue types with a limited number of genes and molecular pathways. Cells accomplish this by reusing the same signaling networks at different times, in different tissues, and for different purposes, yet how this context-specificity is achieved is poorly understood. **Jukam *et al.*** (p. 211, published online 29 August) show how a set of genes that function in cell and tissue growth can be used again in nondividing fly photoreceptor neurons to ensure that flies develop appropriate sensitivity to both blue and green light. The Hippo pathway undergoes a regulatory change—from negative to positive feedback—that requires a tissue-specific transcription factor network. This network uses evolutionarily conserved regulatory factors whose mutations in humans result in degenerative retinal diseases. The context-

appropriate positive feedback in flies ensures an all-or-nothing fate decision necessary to establish a functional visual system.

Two Viruses to Bind

Structural studies of two different H7N9 influenza viruses isolated from humans—A/Shanghai/1/2013 and A/Anhui/1/2013—which have different amino acid sequences in the receptor binding site, provide data indicating that the virus is in transition with respect to host adaptation. The Shanghai virus was one of the first isolated in humans that binds avian receptor glycans with high affinity, but binds poorly to human receptors.

However, the later Anhui isolates can bind both avian and human receptors at high affinity. **Shi *et al.*** (p. 243, published online 5 September) show that four hydrophobic mutations contribute to acquisition of affinity for the human receptor by the virus hemagglutinin (HA) and confirm this effect in binding studies with virus particles. Further comparison of a mutant H7N9 A/Anhui/1/2013 HA with the bird flu H5N1 virus revealed the significance of some of the naturally occurring changes observed in circulating H7N9 viruses, which helps to explain how these viruses have been able to cause many severe human infections in a short time.



RTEL1 in DNA Replication

Genome stability requires the coordinate action of a variety of DNA maintenance systems. The DNA helicase, RTEL1 (regulator of telomere length 1), disassembles recombination intermediates to avoid dangerous by-products. RTEL1 also limits excessive meiotic crossing over and disassembles telomere T loops. **Vannier *et al.***

(p. 239) now show that mammalian RTEL1 is part of the DNA replication machinery. RTEL1 binds to proliferating cell nuclear antigen (PCNA), an interaction that was important for normal DNA replication, replication fork stability, and telomere stability. The RTEL1-PCNA interaction was also critical for protecting cells against tumorigenesis but was not required for telomere T-loop disassembly.

Bacterial Détente?

Type VI secretion systems (T6SS) correspond to dynamic intracellular organelles that are functionally analogous to contractile bacteriophage tails. The T6SS of several bacteria species have been found to be responsible for antagonistic behavior that likely reflects the translocation of toxic proteins (effectors) between cells. *Pseudomonas aeruginosa* is able to sense exogenous T6SS attack and assemble its own T6SS apparatus to launch a retaliatory attack aimed directly at the attacker. Now, **Ho *et al.*** (p. 250) describe how exogenous attack is sensed in a process that involves membrane disruption and suggest that the T6SS provides a general cellular defense mechanism against not only T6SS but also conjugative DNA elements delivered via the type IV secretion system involved in mating pair formation.

BCL11A Variants

Recent chromatin mapping data have suggested that trait-associated variants often mark regulatory DNA. However, there has been little rigorous experimental investigation of regulatory variation. **Bauer *et al.*** (p. 253; see the Perspective by **Hardison and Blobel**) performed an in-depth study of the *BCL11A* fetal hemoglobin-associated locus. The trait-associated variants revealed a chromatin signature that enhanced erythroid development. The enhancer was required for erythroid expression of *BCL11A* and thus for globin gene expression.



Marcia McNutt is Editor-in-Chief of *Science*.

Shutdown!

ON 1 OCTOBER, MORE THAN 800,000 WORKERS IN THE U.S. FEDERAL GOVERNMENT RECEIVED notices that they should not report for work “until further notice.” The federal government is effectively closed for business while the two branches of the U.S. Congress argue about whether a continuing resolution to fund the government until mid-November should defund health care reform in the process. Those laid-off workers include tens of thousands of government scientists deemed “nonessential.”

Although the threat of a government shutdown had been looming for quite some time, I suspect that many scientists who had to abandon their work were not entirely prepared for the reality. It has been 17 years since the last shutdown, also over a medical issue (Medicare). As leader of the U.S. Geological Survey (USGS) during President Obama’s first term, I helped prepare numerous shutdown plans for what has sadly become something of an annual drill, but fortunately I never had to implement any of them. In each case, Congress enacted budget legislation to keep the government funded, even if only minutes before a midnight deadline. But this time, government scientists are literally locked out. The government rules for a shutdown are so strict that many scientists are not allowed to continue their work even as unpaid volunteers. They have no access to their facilities or their government-issued computers. Experiments are interrupted, time series are broken, continuity is destroyed, and momentum is lost.

The entire scientific community will suffer if the shutdown is allowed to endure for any substantial length of time. University and industry laboratories cannot take up the slack: The research portfolios of the science mission agencies have been tuned in such a way that they do not compete with or duplicate the work done in nongovernment facilities. With the vagaries of peer review and the finite duration of grants, it falls to government agencies to maintain critical long-horizon time series and infrastructure such as monitoring networks and observing systems. The science mission agencies have been responsible for much of the applied science done in the public interest; with the shutdown, they will no longer be able to track flu outbreaks, update real-time information on water quality and quantity, improve weather forecasts, develop advanced defense systems to keep us safe, and serve many more immediate needs. Many government laboratories are also involved in translational activities that have not attracted private-sector investment. Regulation, permitting, and oversight of some aspects of research typically fall to the federal government as an essential ingredient for the public good. Without the contribution from government science laboratories, the U.S. research system is greatly compromised.

A shutdown takes a direct economic toll as well. Any organizations waiting for federal grants to be processed are left in limbo and may need to seek bridge funding to pay salaries. For federal employees, the pain is immediate and personal. Paychecks stop. Many federal agencies are already furloughing employees for part of the year to cope with the recent budget sequester. Adding the shutdown to any furlough is a deep hardship for families just making ends meet.

I sincerely hope that this shutdown is resolved quickly and that the impacts are minor. But if not, I urge the research community to take stock of real economic hardships, opportunities lost, and damage done, so as to more effectively argue for congressional action on the federal budget. From my time at the Department of the Interior, I know that one of the most effective cases against federal shutdown was made by the National Park Service (NPS). The NPS was armed with excellent economic estimates of the nationwide ripple effects of closing the national parks on travel and tourism for both domestic and foreign visitors. I hope that the research community can do as well as the NPS.

— Marcia McNutt



EDUCATION

Learners As Teachers

Among emerging massive open online courses, the Peer 2 Peer University remains unique. This online learning platform allows members to take the dual role of learners and teachers: All members are able to create courses, which can be accessed by any online user. To understand how this peer-created, peer-led online environment sustains itself, Ahn *et al.* investigated how members, either as learners or teachers, engaged with open online learning available through the platform. Data describing the nature, history, and activity associated with every project within the platform showed that the Peer 2 Peer University draws a pool of over 40,000 users. However, 85% of members never engaged in the community, and only 18% of the courses were considered to be finished and thus implemented live on the platform. Although the Peer 2 Peer University users actively generate ideas to create courses, they struggle to see these courses through to completion, indicating a need to foster engagement with these teacher and learner users over time. Lack of motivation or community involvement may explain why some teachers leave their projects incomplete. Nonetheless, the data show how crowd-sourced education is useful for small groups with niche interests as well as broad audiences. — FB

J. Online Learn. Teach. **9**, 160 (2103).

GEOCHEMISTRY

Magma Writ Small

Solid phases that precipitate from supersaturated solutions often form from the aggregation or self-organization of molecular clusters or nanoparticles. These undersaturated precursors have been documented in a number of materials for crystals growing from aqueous solution; however, at more extreme conditions, such as in magmatic systems composed of hot melts, evidence has been more circumstantial. Through a series of high-temperature quenching experiments from 950° to 1180°C, Helmy *et al.* demonstrate that Pt- and As-rich nanoscale phases form in sulfidic melts despite being present in low concentrations and far from saturation for macroscopic crystalline minerals. If Pt and other noble metals preferentially form continuums of nanoscale associations with As and related elements instead of homogeneously dissolving in the melt phase, their partitioning will largely depend on surface thermodynamics instead of chemical properties determined by simple partition coefficient experiments. — NW

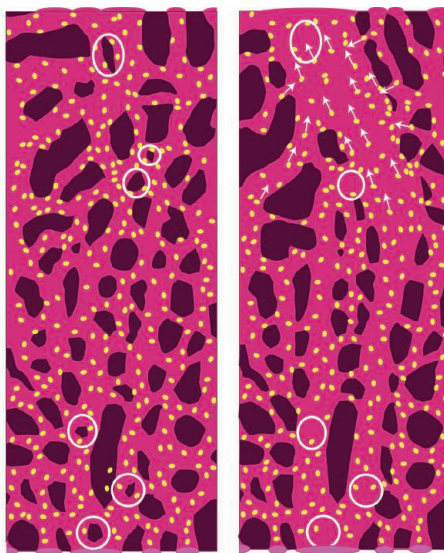
Nat. Commun. **4**, 2405 (2013).

DEVELOPMENTAL BIOLOGY

Growing Vessels

Blood flow in an animal is not only made possible by blood vessel structure, but it also governs the development and remodeling of the vessels themselves. However, little is known about how mechanical forces affect endothelial cells so as to alter vessel diameter. Udan *et al.* have

used time-lapse confocal microscopy to monitor vessel architecture and remodeling in cultured mouse embryos. Larger-diameter vessels contain more endothelial cells, but their proliferation was not affected by blood flow. Furthermore, changes in cell death did not account for vessel diameter variation. Instead, they observed that vessel fusion and directed endothelial cell migration depended on blood flow and that



both contributed to an increase in vessel size. The former occurs near the vitelline artery and vein, where fluid flow is high, whereas the latter involves the migration and recruitment of endothelial cells from capillaries to regions of greater need. This work nicely elucidates the behavioral

biomechanics of cells in vessel remodeling during development. — BAP

Development **140**, 4041 (2013).

EVOLUTION

Sunny Days

The human melanocortin-1 receptor (MC1R) locus in humans is involved in regulating melanin synthesis and hence skin pigmentation; some alleles are associated with lighter skin pigmentation, and several nonfunctional variants are associated with fair skin, red hair, and poor tanning capacity. Furthermore, light skin pigmentation is a risk factor for melanoma. Martínez-Cadenas *et al.* examined the genetic diversity of the MC1R locus in Spain and compared it to those of other populations. MC1R showed high levels of diversity that appear to be due to high CpG content, which are sites of mutation. However, despite the high level of observed mutation, the authors found evidence that most alleles are being removed from the population and are under purifying selection in Spaniards. One exception was the most common variant V60L, which appears to be under positive selection, although it showed spatial heterogeneity. Interestingly, V60L was also the most common variant in a sample of melanoma patients, suggesting that although it may be under positive selection, it has a deleterious and postreproductive consequence as well. — LMZ

Mol. Biol. Evol. **30**, 10.1093/molbev/mst158 (2013).

Continued on page 165

Continued from page 163

BIOCHEMISTRY

Homologous Recombination

In the 1970s, we learned that eukaryotic DNA contains introns—segments that are removed from RNA transcripts by splicing to yield messenger RNA. Since then, introns have been shown to play roles in regulating gene expression. In the 1990s came another surprise, that some proteins contain intervening sequences—inteins, which posttranslationally excise themselves and religate the remnants. Protein splicing usually occurs intramolecularly (cis), with the product comprising two elements (exteins) that were separated by the intein. However, in cyanobacteria, protein splicing also occurs between (trans) two monomer precursors, each containing parts of an intein and an extein. Association of the two monomers completes the intein, and the splicing reaction joins the two exteins. Aranko *et al.* found that splicing can occur between a cis-splicing precursor and a trans-splicing precursor, yielding up to four distinct ligation products. Expressing cis-splicing inteins with an artificially created C-terminal split intein fragment resulted in alternative splicing for all inteins tested, though the ratio of cis to alternative splicing varied. NMR spectroscopy and crystallography confirmed that the alternative splicing occurred through intermolecular domain swapping of inteins. — VV

Nat. Chem. Biol. **9**, 616 (2013).

CHEMISTRY

Multitunable Hydrogels

Hydrogels, consisting of water-swollen cross-linked polymer chains, have proved a versatile platform for creating an artificial environment for cells. Through dynamic crosslinking, it is possible to spatially change the physical and chemical properties within the gel or to allow for drug delivery or degradation of the gel. One challenge is to find routes that allow for either modification of the mechanical properties of the gel or of its local chemistry, without affecting the other. Gramlich *et al.* show that this is possible for a system based on hyaluronic acid, which is a component of native extracellular matrix that has been functionalized using norbornene groups. Gelation occurs through the reaction of the norbornene groups with a di-thiol, but only a few connections were needed to form the gel. Thus, remain-

ing pendant norbornene groups were available for secondary reactions, either to tune the gel's mechanical properties by using additional di-thiol linkages or by using mono-thiol groups to change the local chemistry by linking in additional molecules such as peptides. For example, the compressive modulus ranged from 1 to 70 kPa, depending on the extent of crosslinking for the same overall norbornene content. Using reactions controlled by ultraviolet light made it possible to pattern the gels with either spatial or temporal control of the patterns. — MSL

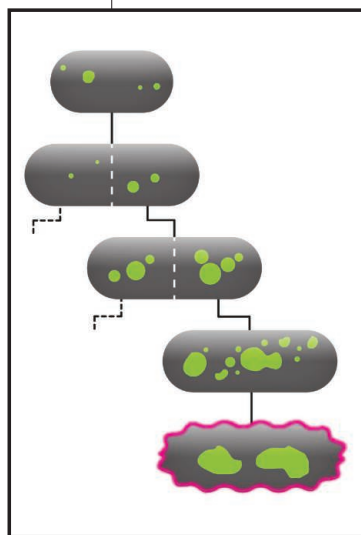
Biomaterials, 10.1016/j.biomaterials.2013.08.089 (2013).

CELL BIOLOGY

Sequestration Stress

All organisms age—at least that's what we thought until now. Although we are familiar with the signs of aging in multicellular organisms

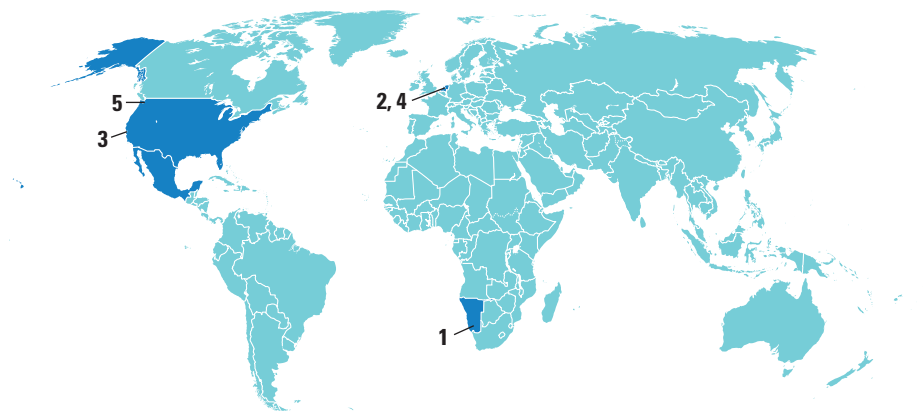
such as ourselves, unicellular organisms also get old though replicative aging, measured as an increase in cell division times and an increased probability of cell death. For example, the budding yeast *Saccharomyces cerevisiae* divides asymmetrically. The larger mother cell ages and normally dies after about 20 divisions. Coelho *et al.* study the fission



yeast *Schizosaccharomyces pombe*, which has rod-shaped cells that divide symmetrically, and show that under nonstressed conditions, neither of the daughter cells or their progeny age—all cells continue to divide at a roughly constant rate. Making the cell divisions asymmetric, producing larger and smaller daughter cells, also did not result in aging. Cells did occasionally die, but death was not preceded by signs of aging and instead was due to catastrophic failure of a cellular process. Under stressful conditions that cause the aggregation of misfolded proteins, fission yeast cells do age. The daughter cell and her progeny, which sequester the single large protein aggregate from the stressed mother cell, will age, whereas the unencumbered daughters will remain ageless. — GR

Curr. Biol. **23**, 10.1016/j.cub.2013.07.084 (2013).

AROUND THE WORLD



Aar, Namibia 1

Namibia Top Choice for Gamma Ray Telescope

A patch of bushy land in southern Namibia is the best candidate to host part of the Cherenkov Telescope Array (CTA), which will be the world's largest gamma ray telescope. Scientists representing the 27-country CTA Consortium met in Warsaw in September, ranking the Namibian site as the best of five options for the CTA's southern array; four sites competing for the northern array earned equal ratings.

Earth's atmosphere obscures direct observations of cosmic gamma rays, thought to be produced by violent astrophysical events

on resolving two mysteries: the origins of cosmic rays, and the nature of dark matter.

The rankings will be forwarded to a 15-nation funding panel that will make the final siting choice; a decision is expected by the end of December, with final approval for the project expected by the end of 2014. <http://scim.ag/CTAsite>

Amsterdam 2

Dutch Challenge Russia Over Arctic Ship Seizure

The Dutch government says it will take Russia to international court over its seizure of a Greenpeace vessel used to stage a protest at an offshore Arctic oil platform. Russian prosecutors last week filed piracy charges against 30 people from 18 nations aboard the ship.

On 19 September, Russian security forces seized the *Arctic Sunrise* near a Gazprom rig in the Pechora Sea northwest of Russia. Greenpeace insists that the protest was peaceful and intended to raise awareness of the environmental risks of Arctic drilling. "Our activists have been charged with a crime that did not happen," said Greenpeace Interna-

tional Executive Director Kumi Naidoo in a statement. On 4 October, Dutch foreign minister Frans Timmermans told reporters that his government will file an arbitration suit at the International Tribunal for the Law of the Sea to challenge the legality of the seizure of the ship, which sails under the Dutch flag.

Among those jailed is former *Science*



Seized. The Greenpeace ship *Arctic Sunrise* anchored outside Murmansk, Russia.

contributor Andrey Allakhverdov, a press officer for Greenpeace Russia. He and the rest of the "Arctic 30" face up to 15 years in prison if convicted.

Mountain View, California 3

'Designer Baby' Patent Concerns Bioethicists

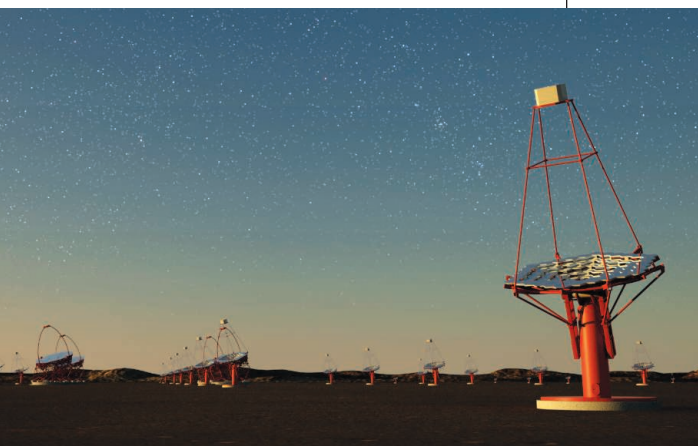
23andMe, the test-your-own-genes company in Mountain View, California, drew criticism from bioethicists last week for patenting a service that predicts the traits of a theoretical child. Critics said it might encourage people to try to conceive designer babies.

Called the "Family Traits Inheritance Calculator," the service is available only to 23andMe clients who permit this use of DNA data. The calculator does not rate disease risks but gives odds on six benign traits, including eye color and muscle performance. In *Genetics in Medicine*, ethicist Sigrid Sterckx of Ghent University in Belgium and three others write that the calculator is "hugely ethically controversial" and that the U.S. government could have denied the patent on moral grounds. Other ethicists said they were not greatly concerned because people didn't appear to be using the service to select embryos or end pregnancies.

23andMe has offered the prediction ser-

NOTED

>Off with that head: New DNA analyses published this week in the *European Journal of Human Genetics* are refuting claims that two famous specimens belonged to French kings: blood from Louis XVI, stored for centuries in an 18th century gourd; and a mummified head thought to be that of his ancestor Henry IV (the Y-chromosome resembled DNA isolated from the blood) (*Science*, 24 May, p. 906). <http://scim.ag/Bourbid>



Looking up. Researchers are rating sites for the Cherenkov Telescope Array (artist's conception shown).

such as supernovas. Cherenkov telescopes, however, detect the flash of light caused by the rays' collision with atoms in the upper atmosphere. The CTA, expected to cost \$270 million and be fully operational in 2019, would be 10 times as powerful as current Cherenkov instruments and will focus

Two Million and Counting

Rare as this carnivorous plant is, the purple pitcher plant *Sarracenia purpurea* has just gotten a lot easier to study. The pitcher plant is the 2 millionth specimen added to the New York Botanical Garden's (NYBG's) massive effort to take high-resolution photographs of the 7 million plant specimens in its William and Lynda Steere Herbarium and digitize them.

The digitization effort began in the 1990s to give Brazilian botanists and others access to information on the NYBG's 450,000 plants from Brazil. At first, only data about the specimens went online, but in the 2000s, NYBG began adding images of the pressed plants, making it possible to study a specimen with the same magnification as under a microscope, says NYBG botanist Barbara Thiers. It took 12 years to digitize the first million plants; 300,000 are now added each year.



vice since applying for the patent in 2008. Company spokesperson Catherine Afarian wrote in an e-mail that 5 years ago “there was some thinking that this feature would have potential applications for fertility clinics,” but that the company “never pursued the idea and has no plans to do so.”

<http://scim.ag/designbaby>

Amsterdam 4

Scientist Falsified Work, CV

A Dutch anthropologist who studied the civil war in the former Yugoslavia engaged in “serious scientific misconduct,” according to an independent investigative panel’s report released on 23 September. The report says that Mart Bax of the Free University made up 64 citations to papers—in journals such as *Current Anthropology* and the *New Mexico Quarterly*—on his CV and in university documents. Bax also embellished his CV with nonexistent guest lecturer positions at Princeton and Cornell and phantom awards.

Bax, who retired in 2002, published dozens of articles about Catholic traditions in the Netherlands as well as fighting between Bosnians and Serbs in 1991 to ’92 in Medjugorje, a village in Bosnia and Herzegovina. The panel determined that his scholarship is riddled with errors and blames Bax for not verifying his informa-

tion and doing nothing to correct statements he knew to be wrong.

The panel also criticizes the Free University’s executive board for dragging its feet on launching an investigation after Dutch science reporter Frank van Kolschooten reported the allegations in a book last year. The university has decided not to take legal action against Bax.

Seattle, Washington 5

Microsoft Co-Founder Hints At Cell Bio Investment

Microsoft co-founder and billionaire Paul Allen appears to have his sights on a cell biology initiative. At a 26 September symposium, he called understanding cell biology “another holy grail” and admitted he



Paul Allen

was interested in developing a dedicated institute, according to a *Puget Sound Business Journal* report. CEO Allan Jones of the Allen Institute for Brain Science says: “Paul is certainly interested in it. ... But beyond that, it’s sort of still in the planning stages.” In August, about 15 scientists and institute

THEY SAID IT

For more tweets on science and the shutdown, visit: <http://news.sciencemag.org/tags/shutdown>

“While the government is shutdown, I can’t even apply for an internship with NASA. Come on politics, I’m just trying to science.”

—Jered Hoff @jered_hoff

“No new experiments. Halted research on deadly brain disease #PML at NIH. Only allowed to maintain cell lines. #essentialscience #shutdown”

—Michael Ferenczy @ViralScience

representatives met to discuss the future of biology research, says computational biologist Sean Eddy of the Howard Hughes Medical Institute’s Janelia Farm Research Campus in Ashburn, Virginia, though no one mentioned plans for an institute. Whether any new project would match the scale of the brain institute—launched with Allen’s \$100 million investment in 2003—is yet to be seen. <http://scim.ag/AllenCellBio>

FINDINGS

Malaria Vaccine Buzzes Forward

The developer of a malaria vaccine that has moved further in clinical trials than any other plans to seek regulatory approval because of promising new results. The vaccine has demonstrated only a modest ability to protect children from malaria, but proponents say data from a study that involves more than 15,000 children in seven sub-Saharan African countries show that it creates durable immunity and could complement other efforts, such as bed nets, to thwart the disease. “The results are encouraging and perhaps more positive than some were expecting,” says Richard Feachem, a malaria specialist at the University of California, San Francisco, who was not involved with the study.

The new data, presented at a malaria meeting in South Africa this week, showed that 18 months after receiving three doses of the vaccine, bouts of malaria dropped by 46% in children between 5 months and 17 months of age. Feachem says he supports the decision by the vaccine’s maker, Glaxo-SmithKline, to file for regulatory approval in 2014. “The sooner we start, the more lives will be saved,” Feachem says.

<http://scim.ag/malariaenough>

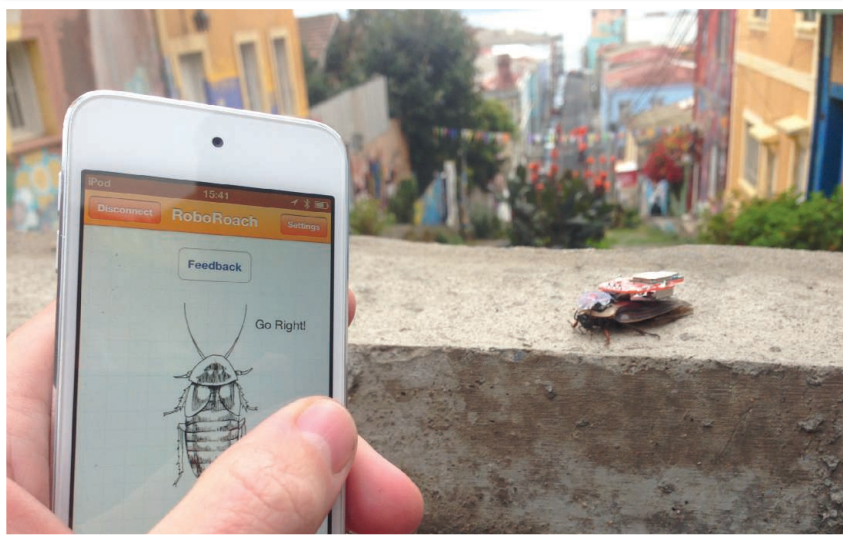
Earning a Good Salary? Thank Your Fourth-Grade Teacher

Students who have even one above-average teacher between the fourth and eighth grades are more likely to attend college and eventually earn more money, notes a study in the *Proceedings of the National Academy of Sci-*



Payout. Good teachers can help you earn more.

ences. Economist Gary Chamberlain of Harvard University analyzed data on more than a million students from 1988 through 2009 at more than 800 schools in a single large urban area in the United States (unidentified



Random Sample

RoboRoach: Imperio!

At the TEDx conference in Detroit last week, RoboRoach #12 scuttled across the exhibition floor bearing a tiny backpack of microelectronics. Its direction was controlled by the brush of a finger against an iPhone touch screen.

RoboRoach is a do-it-yourself neuroscience experiment that allows students as young as 10 years old to create their own “cyborg” insects, say creators Greg Gage and Tim Marzullo, both neuroscientists and engineers, and co-founders of an educational company called Backyard Brains. In November, the company will begin shipping live cockroaches, accompanied by microelectronic hardware and surgical kits, across the nation for \$99.

The roaches’ movements are controlled by electrodes that feed into their antennae and receive signals via Bluetooth signals emitted by smartphones. To attach the device, students are instructed to douse the insect in ice water to “anesthetize” it, sand a patch of shell on its head so that the superglue and electrodes will stick, and insert a groundwire into the insect’s thorax. Next, they must carefully trim the insect’s antennae, and insert silver electrodes into them.

Gage says that the roaches feel little pain from the stimulation, to which they quickly adapt. But critics say the project is sending the wrong message. “They encourage amateurs to operate invasively on living organisms” and “encourage thinking of complex living organisms as mere machines or tools,” says Michael Allen Fox, a professor of philosophy at Queen’s University in Kingston, Canada.

Bioethicist Gregory Kaebnick of the Hastings Center in Garrison, New York, who says he finds the product “unpleasant,” likened it to the forbidden “Imperius Curse” of the Harry Potter novels. The RoboRoach, he says, “gives you a way of playing with living things.”

<http://scim.ag/Roboroach>

for privacy reasons). The data included classroom assignments, test scores in grades four through eight, students’ later college attendance, and their earnings at age 28.

Chamberlain developed a new mathematical model to compare different classrooms within the same school and different classes run by the same teacher. Based only on test results, Chamberlain found, an above-average teacher bumps up a student’s chances of college attendance by less than a quarter of a percent. But taking into account other longer-term data—untested skills teachers impart that help students thrive—a

high-quality teacher sends nearly 1% more students to college. Future work could try to answer why teachers have such a long-lasting impact by looking at other ways of gauging teacher quality, such as evaluations. <http://scim.ag/teacherimpact>

Science LIVE

Join us on Thursday, 17 October, at 3 p.m. EDT for a live chat on **how humans are affecting our own water supplies.** <http://scim.ag/science-live>

PRIZES

Higgs and Cell Studies Nab Nobels

There was little doubt about who would be getting a call from the Nobel physics committee on 8 October. But that didn't make the hourlong delay before the announcement any less excruciating. After last year's hullabaloo over the discovery of the Higgs boson by the Large Hadron Collider (LHC) at CERN, the European particle physics laboratory near Geneva, Switzerland, the laureates just had to include Peter Higgs and François Englert, two of the gaggle of theorists who proposed a mechanism that gives force-carrying fundamental particles mass and that required the existence of the now-famous boson.

But that nail-biting delay spoke volumes: Would they or wouldn't they name a

was "overwhelmed to receive this award." After waiting nearly 50 years since their original papers, "it was worth waiting another hour for," says theorist Frank Close of the University of Oxford.

The prize celebrates a theoretical breakthrough in the mid-1960s when physicists were still piecing together their "standard model" describing the fundamental particles and natural forces other than gravity. The problem is that, at first sight, the theory describes only massless particles, and attempts to include mass made it nonsensical mathematically. Higgs, Englert, and others solved the problem by proposing a new field filling empty space, later dubbed the Higgs field. Particles interact with the field to acquire energy and, hence, mass.

It was Englert and his colleague Robert Brout, also at the Free University, who first published a theory in August 1964. Higgs published his version 7 weeks later. But then in November, a third group—Tom Kibble of King's College London; Gerald Guralnik, now at Brown University; and Carl Hagen, now at the University of Rochester in New York—published

its own version, considered by many the most thorough and complete.

Brout died in 2011, leaving five living researchers with a claim to the prize, which cannot be awarded posthumously. Nobel Prize rules restrict it to no more than three recipients—hence the committee's dilemma. Englert was a shoo-in because of precedence, and Higgs first realized that the Higgs field required its own force-carrying particle. "Higgs made the extra step: a testable consequence," Llewellyn Smith says.

But the other three? "They were scooped," Close says. He thinks Kibble should have been the third laureate because he made further refinements to the theory in 1967 and "had been there throughout [its development]." But Kibble himself says he has no hard feelings. Because of his group's later publication, "it was very difficult to include

us," he says. "But I'm glad they've recognized this topic, as it is a very important one."

Close also thinks this award should be dedicated to "a triumph of engineering": the LHC. The fact that it was built, that it works, and that it did what it was designed for in discovering the Higgs boson should be celebrated. "It is experiment that decides what reality is," he says.

—DANIEL CLERY

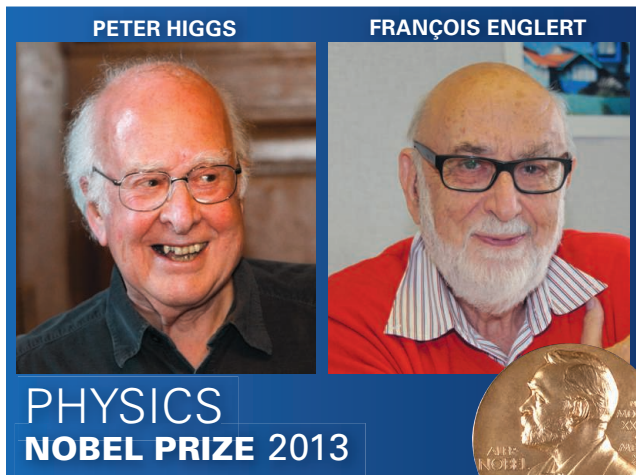
Cell Transport Win Long Overdue

On 7 October, Randy Schekman got the phone call he had long hoped for but thought might never come. At 1:30 a.m. his time, the University of California (UC), Berkeley, cell biologist learned that he was sharing the 2013 Nobel Prize in physiology or medicine with James Rothman and Thomas Südhof for their pioneering work on how cells package and move their hormones, neurotransmitters, and other molecules. "I'd more or less given up, but every year at this time I get anxious," Schekman says.

For years, he and Rothman, a cell biologist at Yale University, had been on many people's Nobel shortlist. Together with Südhof, a Stanford University neurobiologist, "these scientists are really responsible for working out the molecular basis for protein secretion and membrane trafficking," says Suzanne Pfeffer, a molecular cell biologist also at Stanford University in Palo Alto, California. "It's well-deserved and long overdue."

Cell biologists have long recognized that proteins are shuttled through and out of cells using tiny membrane bubbles called vesicles, but until the late 1970s they had little information about the molecular mechanisms involved. Then Schekman and Peter Novick, now a cell biologist at UC San Diego, began to identify mutant baker's yeast strains that still made secreted proteins—but could not release them. The duo identified 23 genes involved in different stages of membrane trafficking. Since then, Schekman's lab team has systematically pinned down those genes' proteins and their specific roles.

Around the same time, Rothman was reconstituting a key cellular component called the Golgi complex in a test tube. Cells shuttle newly made proteins into the Golgi complex, where they are sorted and then dispatched in vesicles to other parts of the cell or to the cell



third laureate, as their rules allow, or even an organization (CERN) alongside the other two? Nobel committees are famously reticent about the debates that go on behind closed doors, but one physics committee member said in an interview that the delay was the result of "a very good discussion." In the end, the committee played safe and stuck with the pair who have an unassailable claim to recognition. "It was the right choice," says former CERN Director-General Chris Llewellyn Smith of the University of Oxford in the United Kingdom.

"You may imagine this is not very unpleasant, of course," Englert, of the Free University of Brussels, told reporters after the announcement. "I am very happy to have the recognition of this extraordinary award." Higgs, of the University of Edinburgh in the United Kingdom, said in a statement that he

surface for secretion. With their innovative cell-free Golgi system, Rothman's team began isolating proteins that help vesicles to pinch off from one membrane and then fuse with a target membrane. One of the first ones they found, called NSF, proved to have a counterpart among the yeast proteins that Schekman was isolating. "There was a moment of epiphany in the field," says cell biologist William Wickner of Dartmouth College. With the discovery of many more such counterparts, "it meant that the whole [intracellular transport] pathway was conserved from yeast to humans."

Next Rothman and Thomas Söllner, now at Heidelberg University in Germany, isolated what Rothman called SNARE proteins. In 1993, Rothman proposed that SNAREs on both the vesicle and target membranes link up via a protein complex that guides each vesicle to the right spot for dumping its contents—a hypothesis that has largely held up.

It turned out that researchers studying neurons, Südhof among them, had SNARE proteins already in hand from their studies of how nerve endings called synapses release neurotransmitters. "Our work surprisingly converged," Schekman says. "We had no idea that we would end up isolating

many of the same molecules."

Many neuroscientists have long expected that Südhof would join the Nobel ranks. "The guy is on a mission: He won't rest until he's got his hands on the last relevant player at the synapse and knows what it does there," says former postdoc Markus Missler, a neurobiologist at the University of Münster in Germany. Born and trained in Germany, Südhof came to the United States to work on cholesterol in the labs of Michael Brown and Joseph Goldstein at the University of Texas Southwestern Medical Center, who received a Nobel themselves in 1985. But after starting his own lab, Südhof used electrophysiology, mouse genetics, and biochemistry to dissect the workings of neurotransmitter release. He identified key protein components that guide vesicles of neurotransmitters to the outer

membrane of a neuron and trigger their release in response to an influx of calcium ions.

The Nobel Prize officially acknowledges that early work, but Südhof has continued to study the precise mechanisms of synaptic transmission. Hundreds of labs now work on molecules that he has identified as important to neural signaling, Missler says, including neuroligins, which may malfunction in autism.

By including Südhof in the award for membrane trafficking, the Karolinska Institute in Stockholm may have preempted a future award devoted purely to synapses, which several neuroscientists say could have sensibly included Reinhard Jahn of the Max Planck Institute for Biophysical Chemistry and Richard Scheller of Genentech, with whom Südhof shared the 2010 Kavli Prize and last month's Lasker Award. Yet no one is quibbling with the choice of the trio. "We have three scientists who were marching to their own drummer, scientifically," Wickner says. "Their work has come together to understand a very fundamental and conserved process [that's] fundamental to human health and disease and to the biosphere."

—ELIZABETH PENNISI AND
EMILY UNDERWOOD

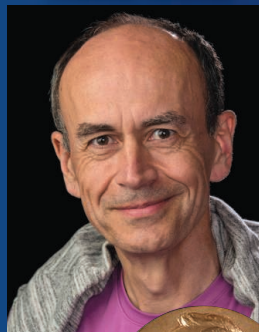
JAMES ROTHMAN



RANDY SCHEKMAN



THOMAS SÜDHOF



MEDICINE NOBEL PRIZE 2013



SCIENCE FUNDING

U.S. Shutdown Tightens Its Grip on Research

Uh-oh, this could get a whole lot worse. That's what many U.S. scientists are realizing as a government shutdown stretches into its second week with no end in sight.

The shutdown, which began 1 October after Congress could not agree on spending for the 2014 fiscal year, immediately idled hundreds of thousands of government

employees, paralyzed grantmaking by key science agencies, and disrupted countless research projects and meetings (*Science*, 4 October, p. 22). But last week, the longer-term peril posed by the crisis came into focus, as some heavily used science facilities began to run out of money.

Astronomers shut down three powerful radio telescopes. The National Science Foundation (NSF) warned that it might have to cancel its upcoming Antarctic research season. And at the 10 national laboratories supported by the Department of Energy's Office of Science, managers warned that academia and industry could soon lose access to synchrotrons and other popular tools. "I don't think we can make it through a month," says Thom Mason, director of the Oak Ridge National Laboratory in Tennes-



Blinded. A lack of government funding forced astronomers to turn off three U.S.-based radio telescopes, including the Very Large Array in New Mexico.

see. “We’re trying to take it day by day.”

The labs didn’t immediately close because they are run for the government by contractors, which have tapped money left over from the 2013 fiscal year. At the Argonne National Laboratory in Illinois, that means the Advanced Photon Source, an x-ray synchrotron that supports 4600 researchers, can operate for roughly another month. Other projects may lose funding sooner.

The rainy day fund didn’t stretch very far at the National Radio Astronomy Observatory (NRAO) in Charlottesville, Virginia, which operates radio telescopes for NSF. On 4 October, it turned off all three of its U.S.-based observatories: the Robert C. Byrd Green Bank Telescope in West Virginia; the Karl G. Jansky Very Large Array in New Mexico; and the Very Long Baseline Array (VLBA) of 10 telescopes stretching from Hawaii to the Virgin Islands. Money for NRAO’s contribution to a multinational project, the Atacama Large Millimeter/submillimeter Array in Chile, could run out in a few weeks, but other countries might take up the slack.

Astronomer Mark Reid of the Smithsonian Astrophysical Observatory in Cambridge, Massachusetts, is part of a team using the VLBA to map the spiral arms of the Milky Way galaxy, relying on twice-yearly measurements, made at opposite ends of Earth’s orbit, to determine distances to stars. If the shutdown doesn’t end by mid-October, in time for the next observing window, he says a year’s worth of data that cost some \$500,000 to collect could become useless.

At the National Institutes of Health (NIH), biomedical researchers got a little good news. After a member of Congress complained that a freeze on an online database was preventing new patients from enrolling in clinical trials, NIH got permission to call back workers to update ClinicalTrials.gov. NIH has also said that it can admit children with life-threatening illnesses to its clinical research center. NSF says it has funds to operate its Antarctic bases and supply systems through 14 October, but will cut back to skeleton crews after that. And if the shutdown continues through 31 October, it must suspend several major science construction projects, including a solar telescope and ecological and ocean observing systems.

Meanwhile, Congress could not agree on a bill that would have given the politically popular NIH a temporary reprieve from the shutdown—evidence of the depth of the impasse.

—DAVID MALAKOFF

With reporting by Adrian Cho, Jocelyn Kaiser, and Jeffrey Mervis.

POLICY

NASA Meeting Bars Chinese Students

Late last month, Yale University postdoc Ji Wang was preparing a poster presentation for a 4 to 8 November meeting that will showcase data from NASA’s Kepler planet-hunting probe when he got word that he would not be able to attend. In a 1 October follow-up e-mail to his boss, astrophysicist Debra Fischer, Mark Messersmith of NASA’s Ames Research Center in California said “federal legislation forbids us from hosting any citizens of the People’s Republic of China.”

In all, NASA has blocked six Chinese scientists from attending the conference, to be held at Ames. The move has enraged the scientific community and caused some to call for a boycott of the conference. And while many scientists have blamed Congress, the policy originated with NASA. The furor has made many scientists aware for the first time how much pressure the agency is facing to show

pending an outside review of NASA security policies, at a 20 March hearing before a U.S. House of Representatives spending panel that oversees NASA’s budget. The panel’s chairman, Representative Frank Wolf (R–VA), is an outspoken critic of China’s human rights record and has also criticized NASA for its vulnerability to espionage.

In 2011, Wolf inserted language into a NASA spending bill that prevents the agency from collaborating with the Chinese government or any government-owned companies and from “hosting official Chinese visitors” at any NASA facilities. That policy triggered a bitter fight between Wolf and the Obama administration (*Science*, 29 April 2011, p. 521), although neither side saw it as applying to individual scientists, much less Chinese students attending U.S. universities. In a letter this week to Bolden, Wolf explains that the law “places no restrictions on activities involving individual Chinese nationals” and says Messersmith’s rejection letter to Wang “mischaracterizes the law and is inaccurate.” Wolf asks Bolden to “correct the record.”

Officials at Ames and at NASA headquarters were not available to comment because of the government shutdown. Meanwhile, many scientists confess that they were not familiar with either the moratorium or the congressional



U.S.A., U.S.A. NASA Administrator Charles Bolden.

language before the current flap. “We simply did not know that this was an issue,” says asteroseismologist Steve Kawaler of Iowa State University in Ames, co-chair of the meeting, which had about 400 people register.

Meeting organizers say they agree with critics that prohibiting attendees by nationality is “deplorable,” but they don’t think boycotting the meeting is an appropriate response. Kawaler would prefer to see the policy issues discussed “alongside the scientific presentations” so that attendees can then spread the word to the broader community.

Ironically, the Kepler meeting was one of the few large conferences that NASA didn’t cancel as a result of budget cuts from this year’s sequester. And Kawaler says that he still hopes to find some way for “our Chinese colleagues” to participate without running afoul of NASA’s moratorium or the law.

—JEFFREY MERVIS

DUAL-USE RESEARCH

Dutch H5N1 Ruling Raises New Questions

AMSTERDAM—In December 2011, the Dutch government asked virologist Ron Fouchier to do something unheard of. Fouchier had shown how a few mutations could make the H5N1 bird flu virus transmissible among ferrets—a finding so serious, the government concluded, that it warranted review as “dual-use” technology that can be used for good or evil.

Before Fouchier could send a second draft of his paper to *Science* in Washington, D.C.—he had submitted a first draft

earlier in the year—the government required him to apply for an export license, as if it were an advanced circuit rather than a research report. Fouchier was outraged about what he saw as a “violation of academic freedom,” but he complied, under protest, and his paper was published in June 2012.

Now it appears that far from being an anomaly, the episode has set a precedent, raising complex questions about how the Netherlands will regulate sensitive biomedical research in the future. Fouchier’s employer, Erasmus MC in Rotterdam, had protested the original requirement and took the issue to court. But in a 20 September ruling, a district court in Haarlem said that government officials had correctly interpreted a 2009 E.U. regulation aiming to prevent the proliferation of nuclear, chemical, and biological weapons when they asked Fouchier to apply for an export license.

Opponents of the research, who support additional roadblocks to what they see as dangerous studies that may help bioterrorists create a pandemic flu strain, welcomed the decision. But experts in nonproliferation say that, whatever you think of Fouchier’s

research, export control isn’t the best way to keep biomedical research in check. “The Dutch government used this as a stopgap measure at the time; it was the only way to get some legal traction on Fouchier,” says Ian Anthony, research coordinator at the Stockholm International Peace Research Institute.

“This has to be taken up at the European level. I don’t see any alternative.”

—IAN ANTHONY, STOCKHOLM INTERNATIONAL PEACE RESEARCH INSTITUTE

papers that caused a worldwide uproar in late 2011 after a U.S. oversight body, the National Science Advisory Board for Biosecurity (NSABB), recommended not publishing them in full. The second was a similar study by Yoshihiro Kawaoka from the University of Wisconsin, Madison.

NSABB’s advice led the U.S. government to temporarily slap export restrictions on Kawaoka’s study as well. But the United States lifted these requirements after NSABB reversed its decision on the two papers in March 2012 (*Science*, 6 April 2012, p. 19). The Dutch government, in contrast, stuck to its position, based on an E.U. regulation prosaically known as 428/2009, which applies to certain bird flu strains, including H5N1.

An annex within the 269-page document makes exceptions for “basic scientific research” and for information already “in the public domain.” Erasmus MC had argued that the study was basic research, and that it used methods already in the public domain.

The court disagreed. Making H5N1 more transmissible was a “practical goal,” the judge said, and while the methods might have been

known, the researchers had “taken steps and made choices that have led to entirely new outcomes.” Any exemptions in the regulation “should be interpreted strictly,” the court said. But legal experts say the ruling raises new questions:

• What does it mean for other Dutch researchers?

The E.U. regulation came with a list of dozens of pathogens for which export rules apply (see table). The Dutch Ministry of Foreign Affairs, in written answers to questions from *Science*, says scientists working on any of these pathogens—unless they’re doing strictly fundamental research—now need to start seeking export licenses as well. It plans to raise awareness of the issue and expects to receive some 100 applications annually.

• What about European journals?

Because the regulation covers exportation outside the European Union, Fouchier says that he can still submit similar H5N1 papers to *Nature*, based in London, or any other European journal. The government disagrees—and it may win that argument, Anthony says. Sending a manuscript to *Nature* in hopes of publishing it worldwide would be akin to shipping nuclear material to the United Kingdom knowing it will be sold to Iran, he says: “I think the Dutch government would have a very strong case to prosecute Fouchier.”

• Does the ruling create a rift between the Netherlands and the rest of the European Union?

Fouchier says the verdict puts him at a disadvantage as a scientist because other E.U. countries don’t appear to interpret the regulation the way the Netherlands now does. Anthony agrees. “If you have 28 different interpretations, the whole law will fall into disrepair,” he says. “So this has to be taken up at the European level. I don’t see any alternative.”

That might happen if Erasmus MC fights the issue all the way to the European Court of Justice, the ultimate arbiter of E.U. law. A “more logical and cheaper approach,” Anthony says, would be if the existing E.U. intergovernmental working group on export control produces a common understanding on the interpretation of the regulation.

Erasmus can appeal the verdict until 1 November; it hasn’t decided whether it will do so. Meanwhile, Fouchier has filed for a new export license, for a follow-up study to last year’s *Science* paper on H5N1. Under protest, of course.

—MARTIN ENSERINK



What's on the List?

E.U. regulations limit the exportation of dozens of pathogens. They include:

Human

32 viruses

E.g., smallpox, Ebola, yellow fever

15 bacteria

E.g., *Yersinia pestis*, *E. coli* O157

4 *Rickettsia* species

E.g., *Coxiella burnetii* (the cause of Q fever)

2 fungi

19 types of toxin

E.g., botulinum toxins, ricin

Animal

17 viruses and 2 mycoplasmas

E.g., Bluetongue virus and some avian flu viruses

Plant

2 viruses, 5 bacteria, and 6 fungi

E.g., potato viruses and a coffee fungus



PSYCHIATRIC RESEARCH

Severe Autism, Often Slighted, Now Targeted for Study

Greg Kapothanasis is 20 years old but has the cognitive ability of a preschool child—and he can't speak. Two years ago, after his school bus ride lengthened to nearly an hour, the 6-foot-tall teenager became increasingly aggressive, lashing out at people, hitting himself on the head, and moving around unpredictably. "The noise really set him off," recalls his mother, Irene. "He just started bouncing off the walls."

Greg has severe autism. His parents had him admitted to Spring Harbor Hospital, in Westbrook, Maine, which specializes in stabilizing such children with acute behavioral problems. Child psychiatrist Matthew Siegel determined that Greg also suffered from an anxiety disorder that triggered his aggression. Clinicians who don't work with severe autism might not know how to assess the mindset of a nonverbal adolescent with the IQ of a toddler. But Siegel knew what to look for.

Siegel's expertise is now being used on a bigger scale. This month, with \$1.2 million in grant funding from the Simons Foundation and the Nancy Lurie Marks Family Foundation, Siegel is spearheading a new consortium dedicated to studying severe autism. Together, the six consortium members—all in-patient facilities staffed by psychiatrists and behavioral specialists—will admit roughly 1000 autistic children in acute behavioral distress per year. By joining forces, Siegel says, "we can maintain a more permanent research infrastructure to accelerate the pace of discovery."

Children who can speak, stay still, and cooperate during testing are overrepre-

sented in studies of autism, Siegel says. Low-functioning children—up to half of all autistic children, by some estimates—have far greater care needs, "but we know virtually nothing about them," says Helen Tager-Flusberg, a psychologist and developmental scientist at Boston University School of Medicine. "If we really want to understand the disorder, we need to look at both ends of the spectrum," Siegel says.

No matter where they land on the spectrum, children with autism share the same core features: trouble relating to and interacting with other people; restricted interests in just a few objects or activities; and a tendency to engage in repetitive behaviors, such as hand-flapping or humming. What sets severely autistic children apart is their lack of verbal and intellectual abilities. These children speak only a few sentences, if at all. According to Siegel, the ability to communicate by age 5 is the number one predictor of later ability to function. "So verbal ability is a hugely important factor," he says. "But we are almost nowhere in our understanding of the difference" between children who can speak and those who can't. This is a key challenge for the consortium.

Characterizing the psychiatric problems that can accompany severe autism is another objective. According to Robin Gabriels, director of the Neuropsychiatric Special Care unit at Children's Hospital Colorado in Aurora (a consortium member), it's often these "comorbidities"—like Greg's anxiety—that set off a child's most troubling behaviors.

Research shows that a third or more of

Making connections. Psychiatrist Matthew Siegel at work with children in protective headgear.

high-functioning autistic children suffer from anxiety, obsessive-compulsive disorder, attention deficits, or major depression, and Siegel thinks these problems may be even more prevalent in severe autism. Psychiatrists normally diagnose these conditions by having a patient respond to questions. Because severely autistic children can't do that, "we have to rely on careful observation" and "translate the [standard] criteria through an autism lens," Siegel says. He aims to develop methods that others can use.

The high-volume, hospital-based nature of the project will give researchers an unprecedented opportunity to observe behaviors such as self-injury, a common problem in this group. Some children hit themselves on the head and break blood vessels, or bite themselves and develop infections. Gabriels says that self-injury can stem from a need for attention and control or a need for sensory stimulation. Siegel adds that lack of verbal ability might also be a factor, but this needs study.

Eventually, the consortium plans to examine genetic differences in the autistic population. The largest genetic database in autism so far—the Simons Simplex Collection at Rutgers University's Cell and DNA Repository in Piscataway, New Jersey, and funded by the Simons Foundation—has samples collected from over 2700 families. But it deliberately excluded nonverbal children with a mental age below 18 months. "What we want now is a better understanding of this severely affected population, both from the genetic and clinical perspective," says Simons Foundation Senior Scientist Alan Packer.

Genetic insights should allow scientists to define subgroups of affected children. "We need to connect the dots between genetic aberrations and clinical presentations," Siegel says. "Do kids with autism that don't speak have a different set of genes than those who do?" Ultimately, he thinks this approach could lead to better-targeted treatments.

Bryan King, director of the Autism Center at Seattle Children's Hospital in Washington, says the new research agenda is long overdue. "This slice of the autistic population is both the most complicated and the most understudied," he says.

Meanwhile, Greg is doing much better, his mother reports. "When we admitted him, he was going 150 miles per hour," she says. "Now he's doing great. He likes to go grocery shopping."

—CHARLES SCHMIDT

Charles Schmidt is a writer in Portland, Maine.

ANCIENT DNA

Farming's Tangled European Roots

Revolutions are rarely simple. Take the spread of agriculture across Europe. About 8500 years ago, the story went, the world's first farmers swept into the continent from the Near East via what is today Greece and Bulgaria. These pioneers of the so-called Neolithic Revolution then took over every corner of Europe, displacing the hunter-gatherers already living there and eventually reaching the far corners of northwest Europe 2500 years later. In this scenario, living Europeans trace much of their DNA back to that incoming tide of Near Eastern farmers.

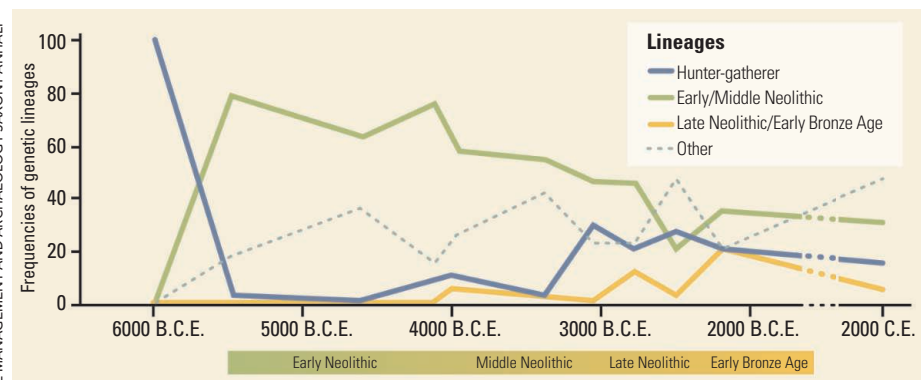
Two papers in *Science* this week use ancient DNA from prehistoric skeletons to

Europeans found clues linking modern Europeans to ancient farming cultures in the Near East. But the genes of living people can't capture the full story. And although more recent ancient DNA studies had suggested a complex transition, those studies focused on local areas (*Science*, 2 October 2009, p. 137).

Now, a team led by paleogeneticists Guido Brandt of the Johannes Gutenberg University in Mainz, Germany, and Wolfgang Haak of the University of Adelaide in Australia present a pan-European view. They built an ancient DNA data set 10 times bigger than any previously studied. They retrieved mitochondrial DNA (mtDNA) from

from the Near East about 7500 years ago. In the second event, beginning about 6000 years ago, central European farmers spread to Scandinavia, where they mixed with hunter-gatherers to give rise to the Funnel Beaker culture. These people were talented animal herders, but also continued to hunt and fish. And in a plot twist typical of the farming story, farmers carrying this mixture of farmer and hunter-gatherer genes then migrated from Scandinavia back into central Europe about a thousand years later, further complicating the genetic picture.

The third event, about 4800 years ago, brought another wave of farmers from the East into Central Europe. And in the fourth, starting about 4500 years ago, metal-wielding farmers of the Iberian Peninsula's Bell Beaker culture flowed into the heart of



Bumpy road to agriculture. The spread of farming across Europe was marked by changes in the frequencies of genetic markers in ancient skeletons, such as this farmer from Germany (right).

decisively upend that simple scenario. One paper, on page 257 of this issue, shows that farming penetrated Europe in a series of fits, starts, and reinvasions, leaving few modern Europeans with the genetic signature of the first farmers. The other paper, published online (<http://scim.ag/Bollongino>), focuses on a single cave that served as a kind of prehistoric catacomb, and suggests that farmers and foragers lived side by side for centuries.

Both papers “contradict the superficial model of European hunter-gatherers being submerged by hordes of farmers” from the Near East, and instead suggest a long and messy transition, says geneticist Martin Richards of the University of Huddersfield in the United Kingdom. The complex pattern of dispersals demonstrates that “ancient DNA is finally fulfilling all the promises we were making for it more than 20 years ago,” he adds. “It’s at last making a reality of prehistoric population genetics” on people no longer around to provide cheek swabs.

Back in the 1970s, researchers seeking genetic signatures of the first farmers in living

364 people from 25 sites in the Mittelbe-Saale region of Germany, an ancient crossroads that was home to at least nine archaeological cultures between 7500 and 3500 years ago. Based on links between bones and artifacts, the team tied specific genetic markers to many of the cultures, mostly farmers and some foragers.

They also added 198 ancient mtDNA samples taken from skeletons in Europe and Asia, plus 68,000 mtDNA sequences from modern Europeans and Asians. The data allowed the group to track the distribution and frequency of mtDNA genetic markers, called haplogroups, through time and space, and to tease out four so-called population events—major migrations that reshaped the genetic landscape of Europe. (Because mtDNA is inherited only from the mother, it reveals a history of women.)

The oldest event was the farmers’ first foray into Europe, when the people of the Linear Pottery culture (LBK), who grew cereals, raised cattle, and crafted distinctive ceramics, surged rapidly into central Europe



the continent. These last events greatly influenced modern genetic diversity, the team concludes. Just 30% of modern Europeans carry the genetic signature of the earliest farmers; signatures from later population movements now dominate (see graph).

Princeton University archaeologist Peter Bogucki questions whether mtDNA can yield such a clear picture of group movements, however. He argues that people may not have moved around in cohesive groups and that small bands of wanderers may have had the cumulative effect of “smearing” genetic signatures over large areas.

Either way, the Neolithic transition appears to have been a prolonged, nonlinear process. It “was accompanied by many trials and errors,” as farmers sometimes learned from indigenous hunter-gatherers how to live in the varying landscapes of the European frontier, Haak says.

Recent studies have suggested that farmers and hunter-gatherers interacted (*Science*, 30 August, p. 950). The second of the new papers uses fresh data from a cave near the city of Hagen in western Germany to show that in some cases, the two groups were long-term neighbors. Excavations at the cave since 2006 have revealed hundreds of human bones from two levels. The oldest, dated between 11,200 and 10,300 years ago, harbored remains of hunter-gatherers from the so-called Mesolithic period. A later level, dated from 5900 to 4900 years old, long after farming was established in the area, was assumed to correspond to Neolithic farmers.

Paleogeneticist Ruth Bollongino of the Johannes Gutenberg University and her colleagues partially or completely sequenced mtDNA genomes from five Mesolithic skeletons

and 20 Neolithic ones. As expected, all the Mesolithic people belonged to an mtDNA group called haplogroup U, typical of European hunter-gatherers. Eight of the Neolithic skeletons showed markers typical of farmers—but 12 were also haplogroup U.

Just who were those “Neolithic” people with apparent Mesolithic ancestry? To find out, Bollongino and her colleagues analyzed the nitrogen and carbon isotopic composition of the skeletons to get an idea of what they had eaten. They got another surprise: While the Neolithic farmers had apparently munched on herbivorous domesticated animals, those with U haplogroups did not. Instead, they ate great quantities of fish. Thus, the team concludes that they were hunter-gatherers, not farmers. “It is very surprising that two culturally different groups used the same burial

site” for 800 years, Bollongino says. “It is difficult to imagine that the two groups did not know of each other.”

Indeed, Bollongino points out, the fisherfolk held onto their distinct lifestyle for 2000 years after the LBK farmers first came to the area. “This study provides the best direct evidence yet for groups with not only different modes of subsistence but also different ancestry coexisting for long periods after the introduction of agriculture,” says geneticist Pontus Skoglund of Uppsala University in Sweden (*Science*, 27 April 2012, p. 400).

Researchers are now busy on the next wave of studies: using ancient nuclear DNA to get an even crisper picture of what both men and women were doing during a revolution that apparently came in fits and starts.

—MICHAEL BALTER

JAPAN

Windfall for Tiny University With Outsized Ambitions

TOKYO—Japan is doubling its bet on a young graduate university, based on a remote island, that has aspirations of becoming a research powerhouse. If approved by the Diet, the annual budget of the Okinawa Institute of Science and Technology (OIST) will jump from \$110 million this year to \$204 million in 2014. OIST’s governors met last week with Japanese Prime Minister Shinzo Abe to outline expansion plans. “We said we admire and congratulate the government for being willing to try to realize the vision for this university,” says neuroscientist Torsten Wiesel, a 1981 Nobel laureate who chairs OIST’s board.

OIST opened for research in 2005 and began taking students last year. Now it intends to double its faculty roll to 100 within 7 years, and ultimately up it to 300—roughly the size of the California Institute of Technology (Caltech). Enrollment is slated to climb from 100 now to 1000. The unabashed hope is “to make OIST the best research university in the world,” says Koji Omi, a politician who conceived the institute a decade ago. “We have momentum now and we should capitalize on that by rapidly beginning to grow,” says OIST President Jonathan Dorfman.

To chart a course for building a formidable institution from scratch, Omi assembled a Nobel-studded team of advisers. They recommended that OIST enroll only graduate students, recruit half its faculty from abroad, and emphasize interdisciplinary research and education. Government planners gave OIST greater autonomy than other universities by placing it directly under the prime minister’s office rather than the education ministry. That privileged treatment riled

Dorfman, a physicist and former director of what is now the SLAC National Accelerator Laboratory in Menlo Park, California. One element, he says, is top-flight faculty members like Keshav Dani, a 34-year-old physicist who studied at Caltech and the University of California, Berkeley. Dani accepted a position at OIST over several tenure-track offers from top U.S. and European universities. When he first visited the campus, he says, “they laid out a fantastic vision and I was blown away.” His startup package included an expensive laser setup for femtosecond spectroscopy.

OIST also has a mandate to spur economic development in Okinawa. Toward that end, Dorfman says, the university in April will establish its first startup company, which will commercialize a technique that determines the molecular structure of proteins developed by OIST biologist Ulf Skoglund. The technique is expected to be used for drug discovery.

Dorfman says he understands why administrators at other universities might envy OIST’s support. But by raising standards of research and setting a precedent for recruiting foreign talent, he argues, OIST is bound to benefit other schools in Japan as well.

—DENNIS NORMILE



Powerful patron. A doubled budget will allow OIST President Jonathan Dorfman, pictured with Prime Minister Shinzo Abe, to plan a major expansion.

some academics and politicians, who also criticized the decision to put the institute in Okinawa Prefecture, better known as a vacation destination than as a research hotbed.

OIST appears to have few detractors now. “We’ve been able to establish the key elements [that] success can be built on,” says



Impact Theory Gets Whacked

Planetary scientists thought they had explained what made the moon, but ever-better computer models and rock analyses suggest reality was messier than anyone expected

LONDON—Among many other contributions to science, the Apollo space program gave geophysicists a grand unified theory of the origin of the moon. The story goes like this: A few tens of millions of years after the birth of the solar system, a now-vanished planet roughly the size of Mars struck Earth a glancing blow that shattered them both and sprayed nearby space with debris. Earth reformed itself; the debris settled into a disk around Earth, which accreted into the moon. The giant impact scenario, based in large part on careful study of the 382 kilograms of moon rocks astronauts brought back between 1969 and 1972, was a triumph of planetary science.

But the truth may not be that simple.

Over the past decade, increasingly sophisticated computer simulations have shown that the tidy scenario clashes with what geochemists have discovered about moon rocks and meteorites from elsewhere

in the solar system. As a result, researchers are casting around for new explanations. At a meeting* at the Royal Society in London last month—the first devoted to moon formation in 15 years—experts reviewed the evidence. They ended the meeting in an even deeper impasse than before, as several proposed solutions to the moon puzzle were found wanting.

So near and—compared with other solar system bodies—so well-known, the moon is not yielding its secrets easily. “It’s got people thinking about the direction we need to go to find a story that makes sense,” says co-organizer David Stevenson of the California Institute of Technology in Pasadena. He and others already see one place that might hold a clue: Earth’s superheated twin, Venus.

Before Apollo, planetary scientists had

put forward various theories of the moon’s formation—for example, that it took shape alongside Earth from the same accretion disk of dust and rubble; that it was a wanderer captured by Earth’s gravity; or that the proto-Earth was spinning so rapidly that it flung into space a blob of material, which condensed into the moon. But each scenario turned out to have some inescapable flaw. Some could not explain the moon’s age, as determined from the Apollo rocks: just slightly younger than Earth’s. Others could not account for the angular momentum bound up in its orbital dance with Earth.

An alternative was waiting: the giant impact hypothesis, first proposed by William Hartmann and Donald Davis in 1975. The scenario appeared outlandish at first, but the

Hit or miss? A planetesimal plowing into the young Earth neatly explains the dynamics of today’s Earth-moon system, but not its geochemistry.

*The Origin of the Moon, Royal Society, London, 23–24 September.

closer researchers looked, the more plausible it seemed. The early solar system swarmed with planetesimals that could have struck Earth. A moderate-speed collision with an impactor about a tenth as massive as Earth would have spewed enough material into space to make the moon while leaving the angular momentum of the new system close to what astrophysicists measure today.

The clincher was the fact that the giant impact scenario also explained three key findings from Apollo: the moon's age, the evidence that it was very hot in its youth, and its chemical similarity to Earth. "It works. You can make a moon," Stevenson told last month's meeting. Scientists embraced the model at a seminal meeting in Kona, Hawaii, in 1984, and, said Jay Melosh of Purdue University in West Lafayette, Indiana, "it worked brilliantly for a decade or two."

Then things started to get complicated. About the same time as the Apollo moon rocks arrived, researchers began studying the ratios of different chemical isotopes in meteorites. The relative abundances of oxygen-16, -17, and -18, in particular, varied so much between meteorites traced to different parts of the solar system that scientists started using the ratios as a marker for the rocks' origin. The moon rocks, however, showed ratios markedly similar to those of rocks from Earth. "The moon and Earth are indistinguishable on the oxygen isotope plot," Melosh said. The isotopes of other elements told the same story.

That didn't trouble geophysicists unduly at first, because they assumed that during the giant impact material from Earth and impactor would be thoroughly mixed. Doubts began to arise in 1986, after a team at Los Alamos National Laboratory in New Mexico published the first computer simulation of the hypothesized collision. The model was crude—it simulated the Earth-moon system with just 3000 particles—but the results were decisive. They clearly showed that after an impact big enough to produce a moon without leaving the Earth-moon system with excessive spin, the moon would consist almost exclusively of material from the impactor.

More recent simulations tell the same story. A 120,000-particle model run in 2004 by Robin Canup of the Southwest Research

Institute in Boulder, Colorado, suggested that the standard giant impact would leave the moon with more than 80% impactor material. This uneven mixing could explain the isotope results only if proto-Earth and the impactor were made of very similar material to start with—a sign, researchers concluded, that they must have formed close together under similar conditions.

That idea received a blow in 2007 from a paper by Stevenson and his then-colleague

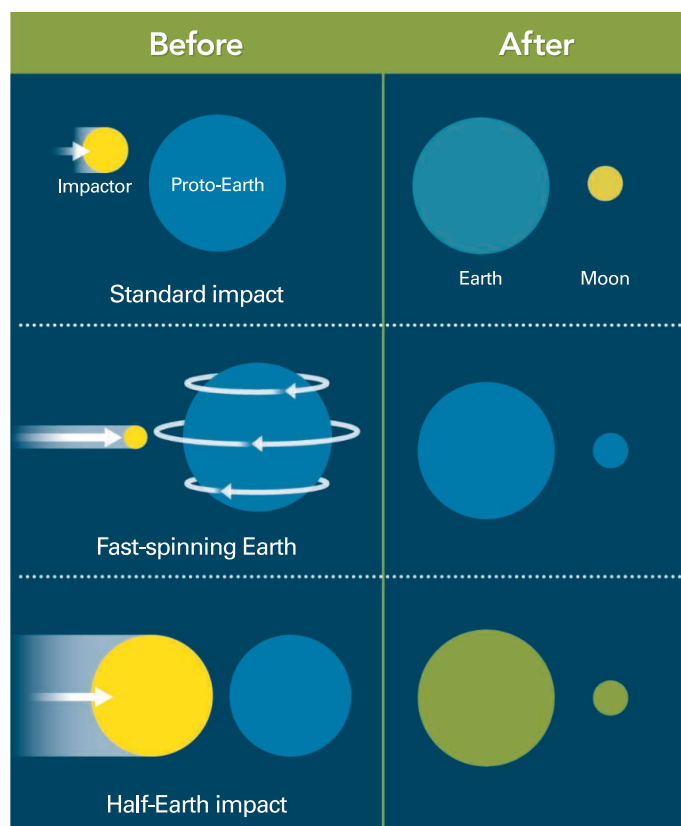
result threw origin-of-the-moon research into disarray, forcing planetary scientists to confront two unpleasant possibilities: either the collision between impactor and proto-Earth was more complicated than they have assumed, or their understanding of the makeup of the solar system needs a major overhaul. "The giant impact has major problems. It doesn't produce the moon as seen," Stevenson told the meeting.

Theorists soon began tinkering with collision scenarios to come up with one that leaves the ingredients of the proto-Earth and its impactor thoroughly mixed. Stevenson and Pahlevan suggested one such scenario in their 2007 paper. They point out that the heat generated by the giant impact would produce an Earth and a debris disk made of molten and vaporized magma. They calculate that this superheated, churning inferno would take between 100 and 1000 years to cool. During that time, they argue, enough turbulent mixing and diffusion could take place between disk and Earth for them to reach an equilibrium, resulting in a homogeneous Earth and moon.

At the meeting, however, some cast doubt on such a scenario. Melosh argued that to reach a uniform composition, Earth and the disk would have to exchange so much material that the disk would collapse. Stevenson admits that this mixing process "can help, but it's not an explanation in itself." Canup says it is important to study the scenario more thoroughly, "but it's a very challenging process to model."

More radically, some want to rethink the whole giant impact scenario. Last year, Matija Čuk and Sarah Stewart of Harvard University proposed that the impactor was far smaller than thought—only about 1/200 as massive as Earth—that it was moving much faster, and that the proto-Earth was already spinning rapidly (*Science*, 23 November 2012, p. 1047). The model can produce a moon of the correct size made up almost exclusively of material blasted from Earth's mantle. Unfortunately, the Earth-moon system winds up with twice as much angular momentum as it has today, but Čuk and Stewart also proposed a mechanism for shedding the excess.

Soon after the moon forms and as the



All mixed up. The standard giant impact (top) has trouble explaining chemical similarities between Earth and the moon. Two models published last year do a better job but leave the system with too much angular momentum.

Kaveh Pahlevan, which modeled how both the impactor and Earth took shape from the disk of debris surrounding the young sun. They argued that a planetesimal like the impactor, with mass a fraction of Earth's, would form out of material from a relatively narrow band around its orbit. A planet the size of Earth, however, would scavenge material from a much wider swathe of the disk, extending past the orbits of Mars and Mercury, where the planet-forming material had very different isotope ratios. So even if the proto-Earth and the impactor formed in similar orbits, their compositions would be different enough to produce distinct isotope ratios in the postcollision Earth and moon.

Stevenson and Pahlevan's unexpected

Earth-moon system is evolving toward its current state, the moon's perigee—the point at which its orbit brings it closest to Earth—moves around Earth in a cyclic motion called precession. The cycles get longer and longer until the rate of precession slows to once per year. Then the precession becomes locked in a fixed position relative to the sun—a rhythm known as evection resonance. As a side effect, the resonance transfers angular momentum from the moon to the sun, in effect spinning up the sun while slowing down the moon. Evection has long been known, but most researchers thought the moon would not stay in the resonance for long. Čuk and Stewart say that in their particular scenario, it could last long enough for the moon to shed half its angular momentum.

In another paper published simultaneously with Čuk and Stewart's (*Science*, 23 November 2012, p. 1052), Canup modified the impact scenario in the opposite way. She showed that a high-speed head-on collision between two bodies with similar masses also could have yielded a homogeneous Earth and moon—again at the cost of leaving the system with too much angular momentum. “Now we can produce a disk with the correct composition, but it still requires [evection] resonance to slow it down,” Canup says.

Evection may prove the Achilles' heel of both scenarios. At the meeting, Jack Wisdom of the Massachusetts Institute of Technology in Cambridge described unpublished results suggesting that Čuk and Stewart had overestimated its effects. When the moon is in the evection resonance, he said, its orbit becomes more elongated, and the change produces extreme tidal forces that heat up the moon. This heating, Wisdom says, would change the moon's physical characteristics enough to end the resonance before it had time to drain enough angular momentum from the system.

Details aside, many researchers at the meeting bemoaned the fact that things are getting so complicated. In the old impact model, a single simple event was all it took to create the moon. Now the models require an impact followed by some other process—



Planetary puzzle. The key to Earth's kinship with the moon might lie in rocks from a more-distant world, Venus.

turbulent mixing or evection—to make it work. “We don't have a single scenario which stands out because of its simplicity,” Canup said. Melosh agreed. “The solutions are contrived; they're not natural,” he said. “We want a solution where isotopic similarity is a natural consequence of the model.”

There is a way out of the dilemma, but it will not be easy to test. The isotopic similarity between Earth and moon would arise naturally in any of the collisions if researchers are wrong in their assumption that isotope ratios vary markedly across the solar system. This assumption stems from meteorites collected on Earth that have been identified as coming from a few other bodies: a couple of asteroids, a comet, and Mars. For example, researchers have some 120 fragments that were blasted off the martian surface by asteroid or comet impacts. Those rocks show very different oxygen isotope ratios from Earth or moon rocks.

But Mars itself is something of an enigma. According to planetary formation models, such a small planet—just 10% as massive as Earth—should not have formed where Mars now sits. So what if Mars actually formed somewhere entirely different and later moved? That would destroy the idea that isotope ratios in the inner solar system

change progressively with distance from the sun. With that constraint removed, it would be much easier for scientists to explain how proto-Earth and its impactor could have wound up with similar compositions.

That explains why at the London meeting, when the session chairs jocularly asked each speaker what single measurement they would most like to perform, many said they would like to examine a piece of rock from the planet Venus. Venus is Earth's rogue twin, and together the two planets contain 80% of the mass between the asteroid belt and the sun. If it turns out that Venus has very similar isotope ratios to Earth, then it is much more likely that an impactor might have had them as well. “Venus is the key,” Stevenson said.

But how to get hold of a piece of rock from Venus? Venus's surface is often described as “hellish,” with atmospheric pressures 92 times those at Earth's surface and temperatures approaching 500°C. Only a handful of probes have survived to reach the surface, and there are no firm plans to return there in the near future. That leaves rocks that fall from the sky: meteorites. Venus's strong gravity makes it much less likely than Mars to have chunks of its rock lofted into space and onto a trajectory toward Earth, but it's not impossible. “We could have a piece in our collections,” Canup says. “But how do we know?”

—DANIEL CLERY

Online

sciencemag.org

Podcast interview
with author Daniel
Clery (http://scim.ag/pod_6155).



Biology's Dry Future

The explosion of publicly available databases housing sequences, structures, and images allows life scientists to make fundamental discoveries without ever getting their hands “wet” at the lab bench

Most life scientists single-mindedly focus their careers on a particular organism or disease—even just a specific molecular pathway. After all, it can often take months of training to master growing a particular cell type or learn a new laboratory technique. Atul Butte, however, wanders from topic to topic—and reaps scientific successes along the way. Though only 44 years old, he has earned tenure at Stanford University’s School of Medicine in Palo Alto, California, based on advances in diabetes, obesity, transplant rejection, and the discovery of new drugs for lung cancer and other diseases.

Butte’s lab is different, too. It isn’t crowded with cell cultures and reagents. His tools look like those of an engineer or software developer: Most often, he’s simply working on a Sony laptop, although at times he does turn to a large computer cluster at Stanford and supercomputers elsewhere when in need of massive processing power. Instead of growing cells and sequencing DNA, Butte, his students, and postdocs sift through massive databases full of freely available information, such as human genome sequences, cancer genome readouts, brain imaging scans, and biomarkers for specific diseases such as diabetes and Alzheimer’s.

Many call this type of research “dry lab biology,” to contrast it with the more hands-on “wet” traditional style of research. Although statistics on the number of dry lab biologists are hard to come by, these data hunters believe they are a growing minority. Butte is one of its top practitioners. Using publicly available data, for example, 2 years ago Butte and his colleagues surveyed the activity of large sets of genes in people affected by 100 different diseases and in cultured human cells exposed to 164 drugs already on the market. By comparing patterns of genes flipped on or off by the diseases and by the drugs, the team drew unexpected connections. They found clues

New miners. New database construction and analysis tools from the iPlant Collaborative (*left*) allow digging through plant and microbial genomes, helping plant biologists around the world improve their understanding of basic biology and advance crop breeding.

that a drug now prescribed for ulcers might also be a useful lung cancer treatment, for example, and that an antiepileptic compound would fight two forms of inflammatory bowel disease (see chart, p. 188). Subsequent lab studies of animals offered support for both inferences. And last month, Butte's group reported in *Cancer Discovery* that a similar approach suggested that the antidepressant drug imipramine would be effective against small-cell lung cancers resistant to standard chemotherapy—a finding that has already prompted the launch of a clinical trial. "This is an exciting time to be doing biological research on a dry bench," Butte says.

And not just for Butte. The growth of publicly accessible data troves on genome sequences, gene activity, and protein structures and interactions has opened new territory for biologists. Seizing on advances in computational power, data storage, and software algorithms able to separate the wheat from the chaff, dry lab researchers are making fundamental discoveries without ever filling a pipette, staining a cell, or dissecting an animal. Thanks to a National Science Foundation-funded initiative called the iPlant Collaborative, for example, there's an emerging generation of data-analyzing "plant biologists" who have never gotten their hands dirty digging in soil or watering seeds. And the National Institutes of Health (NIH) recently announced plans to sink \$96 million into boosting analysis of big data. "There is a transformation happening in biology," says Daniel Geschwind, a neurogeneticist at the University of California, Los Angeles.

"You basically don't need a wet lab to explore biology," agrees David Heckerman, a computational scientist at Microsoft Research in Los Angeles. None of these dry lab biologists believe that advances in data sciences will replace the traditional approach. Rather, they argue that the two dovetail with one another like never before, each propelling the other forward. "I'm like a kid in a candy store," Butte says. "There is so much we can do."

Data for all

Big data is certainly nothing new to science. (*Science* had a special package on the topic in the 11 February 2011 issue.) The Large Hadron Collider at CERN generates 15 petabytes (10^{15}) of data every year it's in operation. Astronomy's Sloan Digital Sky Survey contributes terabytes (10^{12}) yearly as well. Big data isn't even all that new to biology. As of the end of August, for example, NIH's 31-year-old gene sequence database, GenBank, held some 167 million gene sequences containing more than 154 billion nucleotide bases.

Nor is the marriage of computational science and biology novel on its own. Researchers have amassed large-scale basic biology



"I'm like a kid in a candy store. There is so much we can do."

—Atul Butte, Stanford University School of Medicine

data sets for years—unimaginatively dubbed genomics, proteomics, metabolomics, and so on—and combed them in search of novel insights into complex biological pathways and disease.

But many of these early efforts were run by large consortia of researchers, who often had rights to first mine the data before releasing them to the public. So much of that information is now public, however, that it's opened the door for researchers who never participated in those consortia. "Now it's possible to ask big-data questions with data that is extant in the public domain," says Ed Buckler, a research geneticist who specializes in maize genetics at the U.S. Department of Agriculture's Agricultural Research Service in Ithaca, New York, and Cornell University.

Asking those questions requires specialized algorithms and software, capable of handling massive data sets, and those

are improving even as the data proliferate. Heckerman and his Microsoft Research colleagues, for example, made a splash recently with a software advance that eases large-scale searches within genetic databases, such as those used to compare entire genomes in what are known as genome-wide association studies (GWAS). These efforts examine DNA of large numbers of ill people and healthy controls, looking for genetic fingerprints linked to disease. Those fingerprints can be subtle, because most diseases are unlike the simple traits of classical genetics—the colors of Mendel's peas, for example—in which each trait maps to a single gene. "When people first started doing GWAS they thought this would be really easy," Heckerman says. "The problem is that Mendel's peas are the exception not the rule."

Instead, the genetics behind most traits and diseases, such as diabetes and prostate cancer, is far more complex, with small contributions from many genetic changes having an additive effect. "To uncover these weak signals you need tons of data. You need tens of thousands or hundreds of thousands of people," Heckerman says. "But there is a catch. When you analyze lots of data, there is hidden structure," in which separate individuals share a multitude of genetic similarities. But in many cases, these similarities are due to two individuals being more closely related than others, instead of sharing common disease genes. "That wreaks havoc with data. You get tons of what looks like signals. But when you look closer it evaporates."

One way around this has been to use a data analysis approach called a linear mixed model. The approach's mathematical rigor helps reduce false positives, but the computing power needed for it grows as a cube of the number of subjects being analyzed. That's no problem when analyzing a few dozen people or so, but if you want to comb through tens of thousands of genome samples, "forget about it," Heckerman says.

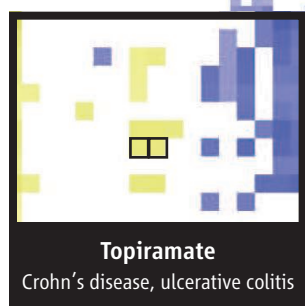
After grappling with the problem for some time, Heckerman and his colleagues came up with what he calls simple "algebraic tricks" to convert the problem to one that scales linearly with the number of subjects, making it tractable to crunch large data sets. The result, an algorithm dubbed FaST-LMM, reduces confounding results, increases the size of the samples that can be processed, and thereby

increases the chance of seeing small signals hidden within large data sets. Last year, Heckerman's team used this FaST-LMM algorithm on Microsoft's cloud-based supercomputer known as Azure to compare the genomes of thousands of individuals in a database run by the Wellcome Trust, a biomedical research charity in the United Kingdom. They analyzed 63,524,915,020 pairs of genetic markers in total, finding a host of new associations that may serve as markers for bipolar disorder, coronary artery disease, hypertension, inflammatory bowel disease, rheumatoid arthritis, and type 1 and type 2 diabetes, as they announced in *Scientific Reports* on 22 January. These associations themselves have been made freely available on the Windows Azure Marketplace so that independent researchers can explore them further.

Butte cautions that such would-be links often fade away upon closer inspection, but he is delighted that software engineers are tackling hurdles in biology. "This is what we have been hoping for," Butte says.

Dry lab biology's impact on biomedicine extends well beyond GWAS studies. Researchers led by Asa Abeliovich at Columbia University, for example, reported in *Nature* on 1 August that they used a big-data approach to discover new molecular actors that influence whether patients with a common variant of a gene known as *APOE4* come down with Alzheimer's. In this case, they used publicly available gene expression data sets from brain tissue of humans with and without a late-onset version of Alzheimer's. They found that two genes, called *SV2A* and *RNF219*,

Drug hit. By analyzing public data on gene expression patterns produced by drugs and diseases, Atul Butte's team identified drugs that might exacerbate diseases (purple) and those that might be therapeutic (yellow). Follow-up studies confirmed that the anti-epilepsy drug topiramate, for example, may treat Crohn's disease or ulcerative colitis.



Diseases

Drugs

have abnormally low activity in people who develop the disease.

Combined with other clues to the genes' functions, the finding suggests that they act as previously undiscovered players in the molecular network that regulates intracellular accumulation of amyloid precursor protein. Amyloid collects in dense plaques in patients' brains and may play a causal role in the disease. Abeliovich's team confirmed the result in lab studies of mice, and then moved on to people—still in a dry lab. The team analyzed publicly available neuroimaging data of Alzheimer's patients and showed that variations in *RNF219* are correlated with the amount of amyloid that accumulates in their brains.

The work not only raises hopes of new drug targets for fighting dementia, but it may also help doctors stratify patients into groups that may one day benefit from different Alzheimer's treatment programs, as they do today for patients with several types of cancer. The experiment, Geschwind notes, was impressive because of the combination of database mining, lab validation, and imaging analysis of now standardized brain scans. "Five years ago they would never have been able to do this," he says.

Beyond biomedicine

The rapid rise in the number of plants that have had their whole genomes sequenced and made public has enabled plant biologists to produce their own dry lab discoveries. Buckler and his colleagues, for example, have been exploring disease resistance across the many species of maize, or corn. In one recent paper, they compared the genomes of 103 different maize species, analyzing 1000 different regions of DNA both within genes and nongene regions of the chromosomes. They linked certain traits, such as variation in disease resistance and in when the plant flowers, to specific patterns of the noncoding DNA. Now, Buckler says, his group and others are helping plant breeding programs improve disease resistance and other traits by singling out which offspring have nongene coding DNA signatures that promote desired traits. "Big data is already having a day-to-day effect on how people are breeding crops," Buckler says.

CREDIT: SCIENCE TRANSLATIONAL MEDICINE/AAAS

It's also helping answer more esoteric questions about plants. David Sankoff, a mathematician at the University of Ottawa, has tapped the whole genome sequences of some 30 flowering plant species to try to reconstruct the general genome architecture—not the specific DNA sequence—of the common ancestor of all flowering plants that lived some 120 million years ago. They recently reported a big step in that direction. By analyzing and comparing the presence of duplicate and triplicate copies of genes found within modern eudicots, one key branch of flowering plants, Sankoff's team concluded that the common ancestor had seven chromosomes and between 20,000 and 30,000 genes, making it a significantly smaller genome than many modern plants. Although such discoveries aren't likely to impact plant breeding or other commercial interests, "it's a really fun aspect of genetics work," says Eric Lyons, a plant geneticist at the University of Arizona in Tucson, who developed a comparative genomics database and software infrastructure used by Sankoff and his colleagues.

Playing well together

Dry lab biology still faces plenty of growing pains. Among the most challenging is gaining access to other people's data. In many cases, researchers who have spent their careers generating powerful data sets are reluctant to share. They may be hoping to mine it themselves before others make discoveries based on their work. Or the data may be raw and in need of further analyses or annotation. "These are really hard problems," Butte says. "We need better systems to reward people that share their data."

A lack of common standards also handicaps the field. Not only do research groups file their data using different software tools and file formats, but also in many cases the design of the experiments—and therefore precisely what is being measured—can differ. Butte and others argue that dealing with multiple file formats is somewhat cumbersome but that the problem is surmountable. But it can be harder to account for differences in experimental design when comparing large data sets.

Years of work to standardize experiments, analysis, and interpretation of experiments involving tools such as DNA and RNA microarrays and proteomic mass spectrometry are beginning to pay off, Butte says. Heckerman agrees. Biological data, he says, are becoming "very standardized."



"You basically don't need a wet lab to explore biology."

—David Heckerman, Microsoft Research

As the volume of publicly available data grows, so do concerns about genetic privacy. Geneticists have shown that even anonymous data can be "reidentified"—and any leaks can reveal not only the medical conditions of patients themselves, but also genetic predispositions to disease that other family members may share. In this case, however, at least one potential solution is already in place. In order to get access to the National Center for Biotechnology Information's database of genotypes and phenotypes (dbGaP), which archives studies such as GWAS associations and molecular diagnostic assays that attempt to link genes to traits, researchers must register and ask for approval. Furthermore, all such requests are made public, so that it's transparent who is attempting to gain access to the data and for what purpose.

To address these challenges—as well as take advantage of the scientific opportunities at the crossroads between big data and biomedical research—NIH announced this summer that it was launching a new project called Big Data to Knowledge (BD2K). With an initial funding of \$96 million over 4 years, BD2K has dual aims. It will establish a series of centers to push the development of novel algorithms and other methodology to make discoveries, and it will also create a series

of working groups across NIH's institutes to deal with the trouble spots of data standards, access, and privacy. Other efforts to grapple with these tough issues exist as well, including a global alliance of more than 70 institutions in 40 countries that was launched in June 2013 to make more digital data freely available.

Dry lab biology could receive a further boost from an upcoming U.S. requirement that databases be open to the community. On 22 February, a memo from John Holdren, the director of the U.S. Office of Science and Technology Policy (OSTP), asked the heads of executive departments and agencies within the federal government to come up with new strategies to encourage access to federally funded science and data. The memo drew attention at the time for its call for increasing open access to scientific publications. But what went largely unnoticed is that the memo also called for digital data from fed-

erally funded unclassified research projects to be stored in publicly available databases. OSTP officials say they have the agency recommendations now and are in the process of reviewing them.

While a potential boon for biology's data miners, access to unprecedented data sources will likely exacerbate problems with data standardization and issues of patient privacy, Butte says. It could also create new headaches for those required to submit their data. They will either have to take time themselves, or hire assistants, to manage the data sets and prepare them for deposition in a public source. And that could take dollars and expertise away from actual research. Particularly in small labs, this may be a significant impact, says Peter Lyster, a program director in the Division of Biomedical Technology, Bioinformatics, and Computational Biology at the National Institute of General Medical Sciences in Bethesda, Maryland. "At some point, it's a zero-sum game."

That's only for the wet labs that generate the data, he adds. For the new breed of dry lab biologists, the combination of new tools, new policies, and burgeoning databases holds nothing but opportunities. Says Heckerman: "I think we're full steam ahead at this point."

—ROBERT F. SERVICE

LETTERS

edited by Jennifer Sills

Retraction

AS A RESULT OF ADDITIONAL EXPERIMENTS IN P.C.R.'S AND S.W.-L.'S LABORATORIES, WE WISH TO retract our 2009 Report, "A type I–secreted, sulfated peptide triggers XA21-mediated innate immunity" (1). Specifically, we have not been able to consistently reproduce the results shown in Figure 3. We have also discovered critical errors in Figures 2 and S3. The strain PXO99 Δ ax21, used in Figure 2, was mixed up with another strain (PXO99 Δ raxSt). When we repeated the experiment with the validated PXO99 Δ ax21 insertion mutant, this strain is still avirulent on Xa21 lines. These results indicate that this insertion in Ax21 does not abolish the ability of PX099 to trigger XA21-mediated immunity. Regarding figure S3, by using more sensitive methods, we have discovered that Ax21 is also secreted in the mutant strains PXO99 Δ raxA and PXO99 Δ raxC. Although we recognize that some parts of this paper may remain valid, we note that key parts of the work depend on the results of Figures 2 and 3. For these reasons, we retract the main conclusion of the paper that a type I–secreted, sulfated peptide triggers XA21-mediated innate immunity.

SANG-WON LEE,¹ SANG-WOOK HAN,² MALINEE SRIRIYANUM,³ CHANG-JIN PARK,⁴ YOUNG-SU SEO,⁵
PAMELA C. RONALD^{6*}

¹Department of Plant Molecular Systems Biotech, Kyung Hee University, Yongin, 446-701, Korea. ²Department of Integrative Plant Science, Chung-Ang University, Anseong, 456-756, Korea. ³Chemical and Process Engineering, King Mongkut's University of Technology, North Bangkok, Bangsue, Bangkok, 10800, Thailand. ⁴Department of Bioresources Engineering, Sejong University, Seoul, Korea. ⁵Department of Microbiology, Pusan National University, Busan 609-735, Korea. ⁶Department of Plant Pathology, University of California, Davis, CA 95616, USA.

*Corresponding author. E-mail: pcronald@ucdavis.edu

Reference

1. S.-W. Lee, S.-W. Han, M. Sririyanum, C.-J. Park, Y.-S. Seo, P. C. Ronald, *Science* **326**, 850 (2009).

Working Together
to Prepare for Disasters

IN ADDITION TO THE DEATHS, INJURIES, AND damage to the physical and economic infrastructure caused by Hurricane Sandy, the storm wreaked havoc on scientific and biomedical research infrastructure. Facilities and specialized equipment were destroyed; thousands of animals, many of which were painstakingly genetically engineered, drowned due to basement flooding; cell lines and laboratory records were lost; and many researchers were left without a place to continue their work. Officials at New York University's (NYU's) Langone Medical Center estimated damages in the range of \$700 million to \$1 billion for rebuilding costs, as well as lost revenue and delayed progress (1).

In response to these losses, M. McNutt and

A. Leshner called for researchers and institutions "to begin discussions, if they haven't already, on how to prepare for the worst, as the worst clearly can happen" ("Preparing for disasters," Editorial, 9 August, p. 592). We propose that individual researchers, research institutions, and research sponsors share the responsibility of improving preparedness to mitigate the effects of future disasters.

Individual researchers must take responsibility for developing an emergency plan that protects their research material and data from unanticipated losses. This includes knowing the circumstances under which research materials, including data, cells, or animals, need to be moved and how they will be moved, as well as how electrical power will be sustained through a disaster. They might also consider collaborative arrangements in which irreplaceable material is routinely stored at a backup facility or another institu-



tion. From the clinical standpoint, researchers could provide their clinical research subjects with a wallet card containing important study information in case participants are lost and need to seek vital treatment. Fortunately, individual researchers do not have to do this alone, and a first step for individuals should be to contact the emergency operations staff at their institutions and request help in completing a detailed risk assessment and with practicing and revising their disaster plan at least annually.

Research institutions are responsible for ensuring not only that the research infrastructure is safe, but also that individual researchers and their labs have a well-conceived and workable disaster plan. Those institutions located in potential flood zones should consider elevating key infrastructure to eliminate single points of failure and to account for new data available from FEMA to assess flood risk over the next 100 years (2, 3). All institutions should have risk-based plans for their research infrastructure that are practiced and updated annually. Several plans and specific suggestions for preparedness efforts are available online (4–6) or from institutions and specific individuals that have addressed past disasters (7–10). Several federal agencies, including the Centers for Disease Control and Prevention, provide guidance regarding how laboratories can be better prepared (11), just as the Joint Commission provides guidance for hospitals (12).

Research sponsors may also wish to consider taking a more assertive role in protecting their research investments by requiring grantees and their institutions to have a risk-based disaster plan in place as a requirement for funding, a requirement that is already in place with certain organizations including the National Institute of Health Office of Laboratory Animal Welfare (13). They could further assist by providing a clearinghouse for model plans or best practices (14).

KRISTIN L. RISING^{1*} AND NICOLE LURIE²

¹Department of Emergency Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA. ²U.S. Department of Health and Human Services, Office of the Secretary, Washington, DC 20201, USA.

*Corresponding author. E-mail: kristin.rising@uphs.upenn.edu

References

1. A. Hartocollis, "A flooded mess that was a medical gem," *New York Times* (9 November 2012); www.nytimes.com/2012/11/10/nyregion/damage-from-hurricane-sandy-could-cost-nyu-langone-millions.html.
2. FEMA, National Flood Insurance Program: Flood Hazard Mapping (www.fema.gov/national-flood-insurance-program-flood-hazard-mapping#00).
3. National Flood Insurance Program, Create Your Flood Risk Profile (www.floodsmart.gov/floodsmart/pages/landing_pages/landing0000_1.jsp).
4. C. M. Vogelweid, *Contemp. Top. Lab. Anim. Sci.* **37**, 52 (1998).

5. J. R. Swearingen, K. J. Vargas, M. K. Tate, N. S. Linde, *Inst. Lab. Anim. Res. J.* **51**, 120 (2010).
6. C. M. Vogelweid, J. B. Hill, R. A. Shea, D. B. Johnson, *Lab Anim.* **34**, 35 (2005).
7. T. Ikeda, *Exp. Anim.* **61**, 1 (2012).
8. S. Matthews, *Nat. Med.* **18**, 1724 (2012).
9. G. Fishell, *Nature* **496**, 421 (2013).
10. B. M. Kuehn, *JAMA* **309**, 123 (2013).
11. J. Y. Richmond, S. L. Nesby-O'Dell, *MMWR Recomm. Rep.* **51**, 1 (2002).
12. The Joint Commission, "Revisions to emergency management oversight requirements" (2012); www.jointcommission.org/assets/1/6/Leadership_Oversight_EM_Proposed_Requirements.pdf.
13. Committee for the Update of the Guide for the Care and Use of Laboratory Animals, *Guide for the Care and Use of Laboratory Animals: Eighth Edition* (Institute for Laboratory Animal Research NRC, Washington, DC, 2011).
14. National Institutes of Health, Grants and Funding, Office of Laboratory Animal Welfare, Disaster Planning and Response Resources (http://grants.nih.gov/grants/olaw/disaster_planning.htm).

A Model of Strength

IN HER AAAS NEWS & NOTES PIECE "CAN the Southwest manage its thirst?" (26 July, p. 362), K. Wren quotes Ajay Kalra, who advocates a particular method for predicting Colorado River streamflow "because it eschews complex physical climate models for a statistical data-driven modeling approach." A preference for data-driven models may be

appropriate in this individual situation, but it is not so generally.

Data-driven models often come with a warning against extrapolating beyond the range of the data used to develop the models. When the future is like the past, data-driven models can work well for prediction, but it is easy to over-model local or transient phenomena, often leading to predictive inaccuracy (1).

Mechanistic models are built on established knowledge of the process that connects the response variables with the predictors, using information obtained outside of an extant data set. One may shy away from a mechanistic approach when the underlying process is judged to be too complicated, but good predictive models can be constructed with statistical components that account for ingredients missing in the mechanistic analysis. Models with sound mechanistic components are more generally applicable and robust than data-driven models.

DOUGLAS H. JOHNSON^{1*} AND R. DENNIS COOK²

¹U.S. Geological Survey, Northern Prairie Wildlife Research Center, University of Minnesota, Saint Paul, MN 55108, USA. ²School of Statistics, University of Minnesota, Minneapolis, MN 55455, USA.

*Corresponding author. E-mail: douglas_h_johnson@usgs.gov

Reference

1. D. J. Hand, *Stat. Sci.* **21**, 1 (2006).

The Editor's Dilemma

IN HER EDITORIAL ON "RISK" (12 JULY, P. 109), M. McNutt confronts a dilemma shared by reviewers of grant proposals and journal manuscripts: how to remain skeptical, yet open-minded. Implicit in her commitment "to

make good decisions in risky matters" is the structure of the review process. But this must be made explicit: How can participants in the review process control or balance biases to achieve fair decisions? To be human is to be biased, as research on "implicit" or "unconscious" bias demonstrates (1). That is where the judgment of editors and program officers is ultimately tested: How do they weigh the recommendations of reviewers who view risky ideas through intellectual lenses colored by knowledge, experience, and their

own competitive juices? In short, "examining the evidence with an open, unbiased mind" does not suffice. The structures implemented under the umbrella of "peer review" must work to subject those individual reviews to something that editors present as a defensibly collective decision (2), one that authors and reviewers alike deem acceptable.

DARYL E. CHUBIN

Savannah, GA 31405, USA. E-mail: daryl.chubin@comcast.net

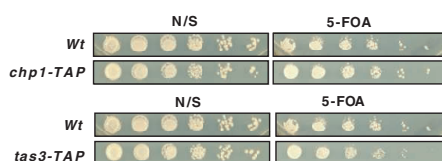
References

1. C. A. Moss-Racusin, J. F. Dovidio, V. L. Brescoll, M. J. Graham, J. Handelsman, *Proc. Natl. Acad. Sci. U.S.A.* **109**, 16474 (2012); published online 17 September 2012.
2. Peer Review at *Science* Publications (www.sciencemag.org/site/feature/contribinfo/review.xhtml).

CORRECTIONS AND CLARIFICATIONS

News of the Week: "Findings: Just a cup for pregnant moms?" by E. Underwood (9 August, p. 597). The article incorrectly states that research by Silva and colleagues on the effects of caffeine on fetal brain development in mice was performed at the University of Coimbra in Portugal; the research was done at INSERM in Marseille, France. In addition, the study addressed not just one, but several subtypes of GABA neurons, some of which decreased in number in adult caffeine-exposed mice. The HTML version online has been corrected.

Reports: "RNAi-mediated targeting of heterochromatin by the RITS complex" by A. Verdel *et al.* (30 January 2004, p. 672; published online 2 January 2004). Levels of growth on medium (Fig. 1B) contained a misplaced photograph. The row of wild-type (*Wt*) cells in nonselective medium (labeled N/S) should have been the same as the row of N/S *Wt* cells in Fig. 2B but mistakenly showed N/S *tas3-TAP* cells from Fig. 2B. The corrected Fig. 1B and, for comparison, the original Fig. 2B are presented here. The conclusions were not affected.



Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to www.submit2science.org.

PHARMACEUTICS

Artemisia, Malaria, and the Red Queen

Wendy Lawley, Caroline Calvert, Ian A. Graham

The Chinese medicinal plant *Artemisia annua* has been a popular choice for relief of fever for centuries, and the

World Health Organization (WHO) recommends artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated malaria caused by *Plasmodium falciparum* (2). A shortage in the availability of artemisinin in late 2004 (3) led the U.S. Agency for International Development (USAID) to support *Artemisia* cultivation in Africa. It was in that context that agricultural economist Dana Dalrymple prepared a USAID briefing paper that has subsequently grown through frequent revisions into the e-book *Artemisia annua, Artemisinin, ACTs and Malaria Control in Africa*. As with its predecessors, Dalrymple has released the work without copyright and made it freely available online. In its most recent version, the book incorporates selected updates through to March 2013.

At present, about 90% of the deaths due to malaria occur in WHO's African region (4). So it is appropriate that the book, in an informal style, provides an accessible introduction to malaria control from the African perspective. Although he focuses on Africa, Dalrymple notes that *Artemisia* has long been a traditional remedy for a range of ills (including malaria) and that during the 1970s Chinese researchers demonstrated the qualities of the purified extract artemisinin. He rightly acknowledges the act of "global public good of the first order" the Chinese performed in sharing their knowledge with the world.

Dalrymple combines an overview of the scientific literature with a repository of per-

sonal communications and contributions from members of the *Artemisia* community. He provides readers with a broad history that

spans the discovery of artemisinin, efforts to encourage *Artemisia* cultivation in Africa, and initiatives to increase the availability of ACTs in countries where malaria is endemic. His account sometimes reads like the collective memoirs of a community, and in places it feels like a working document awaiting updates.

Yet this approach offers a fascinating insight into how this particular subject has unfolded.

The author cites over 1400 references and relates some of the key developments from research between 2006 and 2012—notably semi-synthetic artemisinin and the

commercial availability of high-yield hybrid *Artemisia* seed. He does not, however, set out to provide a definitive review of specialist scientific publications in the field. As one might expect given his background, Dalrymple focuses not on the specifics of scientific advancement per se but rather on the sometimes complicated interplay among public policy, economics, and social science in using these advancements to improve public health. He stresses the importance of placing research results within a holistic approach to malaria prevention and cure. Acknowledging the complexities therein, he calls for "individuals with, or representing, a more comprehensive view of malaria control" to address the "epidemiological, medicinal, and macroeconomic issues" he has identified.

The book raises conundrums in the use of *Artemisia* and artemisinin for readers to ponder. Dalrymple does not provide answers; rather he notes that "[i]n this sense, this paper ends up not far from where it started." *Artemisia* was originally a Chinese medicinal herb, harvested from the wild and administered as a tea by local communities. Dalrymple suggests that its necessary transition to a regulated, commercial product (ACT) raises complexities in intellectual-property management of "impure public goods, ones that incorporate both public and private dimensions." Furthermore, he quotes scientists who question a reliance on plant products in 21st-century society while acknowledging both the importance of *Artemisia* agriculture in Africa and the opportunities presented by natural products and their derivatives in addressing health care needs. Dalrymple provides readers with sufficient insight to become frustrated by the many challenges associated with achieving effective malaria control and stabilizing the ACT supply chain, but he often leaves them to form their own conclusions about the possible solutions.

Artemisia annua, Artemisinin, ACTs and Malaria Control in Africa has far outgrown its humble origins and perhaps is now best considered as an *Artemisia* community record, albeit one that continues to rely on Dalrymple to provide coordination,

Artemisia annua, Artemisinin, ACTs and Malaria Control in Africa:

Tradition, Science and Public Policy

by Dana G. Dalrymple

Published by the author, 2013. 274 pp. \$18.95. ISBN 9780615615998. (1)



Original source. *Artemisia annua*.

The reviewers are at the Centre for Novel Agricultural Products, Department of Biology, University of York, York YO10 5DD, UK. E-mail: ian.graham@york.ac.uk

perspective, and expert comment. The book also provides a thorough introduction to the challenges of malaria control—which Dalrymple likens to the advice the Red Queen gives Alice in Lewis Carroll's *Through the Looking-Glass*: “Now, here, you see, it takes all the running you can do, to keep in the same place.” If the book encourages readers (especially specialists) to consider new, multidisciplinary approaches to tackling malaria, Dalrymple will consider his continued efforts a success.

References and Notes

1. Available at www.mmv.org/newsroom/publications/artemisia-annua-artemisinin-acts-malaria-control-in-africa.
2. www.who.int/malaria/publications/world_malaria_report_2012/en/.
3. www.who.int/mediacentre/news/releases/2004/pr77/en/.
4. www.who.int/malaria/world_malaria_report_2011/en/.

10.1126/science.1243594

HEALTH CARE

Behind What Doesn't Make Sense

Richard S. Mathis

I often tell my children to be careful when they interact with the health care system, because it is not always rational. Tests may be ordered that aren't necessary, procedures recommended that are harmful, and mistakes made that could have been prevented.

You would think that health care in the United States would be very different. Isn't it delivered by highly trained, intelligent professionals who have years of education and sizable resources at their disposal? Yet we Americans can hardly call our health care system a model of efficiency and rationality. Although 18% of our gross domestic product is spent on health care, our health outcomes do not compare favorably with those of other developed countries. We are typically close to the bottom of the list on measures related to mortality and morbidity.

It is tempting to attribute the shortcomings of our system to a single villain. Private insurance companies, the government, and pharmaceutical manufacturers have all had

The reviewer is at the Health Institute, BlueCross BlueShield of Tennessee, 1 Cameron Hill Circle, Chattanooga, TN 37402, USA. E-mail: rsmathis@me.com



their turns. But what if the problem goes a little deeper? What if it involves such factors as the way individuals make decisions? Douglas Hough's *Irrationality in Health Care* provides an interesting perspective on the topic. Hough (an economist at Johns Hopkins University) uses the tools of behavioral economics to understand why certain areas of irrationality exist. This fairly new school of economics differs from the mainstream, neoclassical approach, which assumes that people are rational and act in their own best interests. Behavioral economics draws on contemporary psychology and acknowledges that people are often not rational. People don't always try to maximize their happiness through a utility function that enables them to choose wisely among available goods and services.

Irrationality in Health Care
What Behavioral Economics Reveals About What We Do and Why

by Douglas E. Hough
Stanford University Press,
Stanford, CA, 2013. 311 pp.
\$39.95. ISBN 9780804777971.

Instead, their decisions are often made based on trial and error and a current situation that acts as reference point. Purchasers buy differently, for example, if they are “shown a more expensive house before a less expensive house, a fully equipped car before a stripped-down model, a fifty-two-inch LCD [liquid crystal display] television before a more modest set.”

Hough constructs his consideration around a list of 23 anomalies in health care that are not rational and, therefore, cannot be explained by neoclassical economics. Some of these anomalies cover issues that people in health care frequently wonder about—such as why the public supports specific aspects of health care reform but not the Patient Protection and Affordable Care Act containing those provisions, why patients insist on getting a prescription when they visit a physician, and why doctors can take a long time to adopt new treatment regimens and checklists. Other anomalies spotlight some of the most controversial issues

in medicine, such as why there was an uproar in November 2009 when the U.S. Preventive Services Task Force recommendations for screening mammography were released.

Behind such anomalies are quirks in the way that people tend to think and feel. For example, people tend to fear loss more than they appreciate gain and to overreact to the prospect of getting something for nothing. Thus, although they may support such Affordable Care Act provisions as prohibiting

insurance companies from denying coverage due to preexisting conditions or setting lifetime limits on the total amount paid out for an individual's health care, they fear that the law will reduce coverage and take away services they perceive to be free. These factors tend to trump those driving support. The same is true of establishing guidelines that appear to limit a woman's receiving mammography screening below 50 years of age. Even though the guidelines clearly state the rationale for the recommendation, the public outcry was deafening.

Other tendencies that can influence decision-making include action bias, poor decision-making when faced with too many choices, and overoptimism when remembering compliance with rules and recommendations. Such inclinations contribute to patients insisting on prescriptions, consumers finding it difficult to reach a decision when provided with several health insurance options, and physicians misremembering how many times they washed their hands throughout the day.

Hough carefully avoids using behavioral economics as an explanation of all things rational and irrational in U.S. health care. He is also realistic about the current status of behavioral economics as a “young and imperfect science.” Recognizing the limitations of the advancing field, he does an excellent job of applying it to well-known conundrums. The book could have been improved had the author listed all of the factors that contribute to irrationality in one place. That would have been a better use of space than including in the book the text of his interviews with physicians and economists. Although meant to shore up his points, those prove somewhat distracting. My minor criticisms, however, should not dissuade either health care practitioners or members of the public from reading *Irrationality in Health Care*. Both will find the book well worthwhile.

10.1126/science.1244720

MEDICINE

Newborn Screening: Gaps in the Evidence

Bridget Wilcken^{1,2*}

This year marks the 50th anniversary of mass newborn screening. Newborn screening is becoming more important, with new treatments, new technologies for detection of marker compounds, and cheaper ways to sequence the genome. But it has also become more controversial, demanding renewed discussion about the aims of newborn screening and whether it should still be directed, as at present, to ameliorating only disorders manifest in childhood.

In 1963, Guthrie published a method for testing newborns for phenylketonuria (PKU), a rare, recessively inherited enzyme deficiency usually resulting in severe mental retardation unless treated from early infancy (1). His bacterial inhibition assay involved testing blood samples taken by heel-stick and dried on filter paper. The method was simple. Samples could be sent to a central lab for testing. During 1963, he tested >400,000 samples and detected 37 cases of PKU. The sensitivity was good, and most children received dietary treatment by 1 month. The feasibility of mass screening was shown (2).

The next important step was development of a method of screening for congenital hypothyroidism (CH), in 1975 (3). Testing for PKU and CH was widely adopted in most countries with developed health systems. Individual tests for a few other rare disorders were added in various jurisdictions. However, there was little or no systematic assessment of benefits and risks of screening.

In the 1990s, a pivotal change occurred with development of electrospray ionization tandem mass spectrometry (MS/MS). Many biochemically related disorders could be detected in one test, which allowed screening for disorders that might not have been deemed suitable for separate testing because of their rarity (4). Another important advance was the realization that filter-paper blood samples are an easy and efficient source of DNA. Newborn screening became of much greater interest to pediatricians; scientists; and, in the United States, parent groups, who

perceived that newborn testing was something of a lottery: which disorders were tested depended on the state where the birth took place.

It is possible now to screen newborns easily and cheaply for >50 rare disorders, and the number will increase quickly. The most universally accepted criteria for screening are the 10 principles of Wilson and Jungner (5), summarized as follows: The condition should be an important problem with known natural history, and have an agreed policy on whom to treat as patients, and diagnostic and treatment facilities should be available; there should be a suitable, acceptable test; and the cost of case-finding should be economically balanced in relation to medical costs as a whole. These criteria are sound, but there are new problems with newborn screening.

The Problems

There is a lack of evidence about screening for most disorders (6). For PKU and CH, there have been no formal trials, but there is much evidence of the effectiveness of screening in preventing disability. The only randomized controlled trials of screening were two conducted for cystic fibrosis (CF) starting in 1985, one clearly suggesting benefit (7). After screening programs for CF began in the early 1980s in New Zealand and regions of Australia, France, the United Kingdom, and the United States, the evidence of benefit from early diagnosis and intervention mounted inexorably (8). Children diagnosed early by screening and treated showed nutritional and growth advantages, less morbidity, and some evidence of improved lung function. In 2004, the U.S. Centers for Disease Control and Prevention advised screening: "The magnitude of the health benefits from screening for CF is sufficient that states should consider including routine newborn screening for CF" (9). Yet, in 2006, CF was barely included in the list of disorders recommended to U.S. screening panels, as it fell just above the cut-off threshold and behind 36 other disorders, some vanishingly rare and with no evidence to support screening (10). Not until 2010 was screening universal in the United States, United King-

Despite improvement and increased testing, standards and practice in screening babies for rare disorders remain muddled.



dom, and 10 European countries. It is not yet universal in Canada.

For MS/MS screening for disorders of amino acid and fatty acid metabolism, there have been few reports quantifying benefit and only one with both historical and contemporaneous control groups. That study of 2 million births either screened or not screened, was still too small to confirm benefit in any of the rare disorders individually, except for medium-chain acyl-CoA dehydrogenase deficiency (MCADD), although it did demonstrate an advantage if all other disorders were combined (11). This illustrates how rarity makes assessing benefit difficult.

Another major reason for the difficulty is that far more cases are detected by screening than are detected clinically among patients presenting for medical attention with symptoms. Groups of screened and unscreened patients are not comparable: Screened groups may include those with milder cases, possibly never destined to become symptomatic; unscreened groups may include never-diagnosed deceased patients. Comparisons must be between whole populations, not simply between screened and unscreened affected subjects, which greatly reduces statistical power.

Also, definitions of disorders have not been adequately developed to enable like to be compared with like; the natural history of some disorders, especially of the milder phenotypes, is not well known. Screening for lysosomal storage disorders has uncovered a surprising number of babies whose posi-

¹The Children's Hospital at Westmead, Westmead, New South Wales 2145, Australia. ²The University of Sydney, Sydney, New South Wales 2008, Australia.

*Corresponding author. bridget.wilcken@health.nsw.gov.au

tive screening results do not differ from those of clearly affected cases, but who remain asymptomatic. The significance or probable age of onset of symptoms in such cases is unknown (12).

The harms likely from newborn screening largely relate to the worry caused by false-positive results or, worse, results of uncertain significance—which leave parents in limbo, uncertain whether or not their child is affected—and to overmedicalization in cases where treatment is not needed, with attendant anxiety and costs of unnecessary clinical care. These are not trivial issues and are sure to increase if, in the near future, newborns are screened by whole-genome, exome, or more targeted genetic sequencing.

In considering which screening tests to offer, different countries have come up with widely divergent practices (13). The United States has adopted a liberal approach, with an increasing number of disorders being recommended to states for inclusion. The original list included some disorders currently considered benign, but the recommendation process is now rigorous (14). However, pressure from parent groups directed at individual states has resulted in some screening for nonrecommended disorders.

The results of advocacy group pressure can be seen in the history of screening for Krabbe disease (globoid-cell leukodystrophy), a lysosomal storage disorder of young infants causing progressive neurological dysfunction and death usually by 2 years of age. A 2005 report of improved mortality after hematopoietic stem-cell transplantation was performed presymptomatically resulted in pressure from an advocacy group in New York state to start newborn screening. Screening began in 2006, contrary to advice from experts, and was later rejected by the U.S. Advisory Committee on Heritable Disorders (14).

Among the first million babies screened, of the 228 who initially tested positive, further testing indicated 114 to be unaffected; 84 at low risk; and 26 at moderate or high risk of being affected, with low enzyme activity, some having two mutations in the *GALC* (“Krabbe”) gene. All have so far remained asymptomatic. Of four considered definitely affected, one died having refused treatment, one had some symptoms at transplant and did poorly, one died during the treatment (expected to have about a 15% mortality rate), and one was treated and is doing well, but with some symptoms (15). The cost of screening and confirmation was \$3.5 million. A review of the outcome of 25 U.S. children who received transplants presymptomatically (most identified as affected siblings) showed

that all developed progressive neurological deterioration despite transplantation (16).

The United Kingdom, by contrast, has adopted a very cautious approach. MS/MS screening for one disorder, MCADD, undertaken as a pilot study in 2005, became routine in 2010. A further five disorders, including glutaric aciduria type 1 (GA 1), are being studied in a pilot program starting in 2012—up to 14 years after such screening became routine in some large jurisdictions (17). Overall, in Europe, 13 of 37 countries currently use MS/MS in newborn screening, and include between 2 and 29 different disorders.

All approaches contain ethical dilemmas. The U.S. system maximizes harms from unnecessary detection, with some increase in benefit from early diagnosis. The UK approach minimizes harms arising from screening, while allowing quantifiable harms from not screening. As an example of the latter, GA 1 is a devastating disorder usually leading to death or severe irreversible neurological damage. Dietary and emergency management of intercurrent illness during the first years is highly protective (18). But around eight UK children per year will have died or suffered irreparable damage during each of 10 years when screening was not performed. The cost of screening for this particular disorder is tiny.

Policy Implications and Recommendations

Randomized controlled trials in newborn screening are almost impossible. Rare disorders require huge multicenter trials and prolonged recruitment, as well as prolonged follow-up, as some disorders may have effects in adulthood. Policy-makers must accept good-quality, lower-order evidence. However, the main policy implication is that follow-up and assessment must be built into all screening programs and adequately funded. Costs for screening one baby are small, but for the whole newborn population, screening is an expensive undertaking. It is important to know that the money is well-spent.

It is impossible to be sure that screening for a particular disorder is beneficial until it has been tried, as shown with Krabbe disease. Introducing new testing as research, not mandated, and asking for separate consent from parents (i.e., the baby receives routine screening and the parents can opt whether or not to have the extra research tests) have been undertaken successfully (19). This is the only solution to allow beneficial testing to be adopted. Mandatory testing is not usual in newborn screening outside the United States, but very high levels of coverage—usually more than 99%—are routinely achieved. It may be time for the United States to revisit mandated screening.

One test detecting many disorders simultaneously introduces problems. Benefit may seem clear-cut for one disorder, but for others, detected in the same test for almost no added cost, there may be more harm than benefit. If, in addition, some screening is to be performed as research, more parent and public information will be needed.

Newborn screening has revealed difficulties in distinguishing severe early-onset phenotypes from adult-onset or benign ones. This dilemma is likely to become more marked, especially if primary DNA testing is used. We need to explore whether the aim of newborn screening should be extended to encompass late-onset disease or to remain strictly, as now, to ameliorate problems that will arise in infancy and childhood. In addition, although public advocacy is important, often valuable, and raises matters deserving of consideration, these groups' concerns must not be allowed to overtake science. It is, for example, arguable whether it is a benefit to convert an early lethal disease to a chronic progressive one as has happened with Krabbe disease.

Newborn screening has been a success story, but all proposed advances must be approached thoughtfully, or there could be public health disasters waiting around the corner.

References and Notes

1. R. Guthrie, A. Susi, *Pediatrics* **32**, 338 (1963).
2. R. Guthrie, S. Whitney, “Phenylketonuria detection in the newborn infant as a routine hospital procedure” [Publication 419, U.S. Department of Health, Education, and Welfare (HHS), Washington, DC, 1964].
3. J. H. Dussault *et al.*, *J. Pediatr.* **86**, 670 (1975).
4. D. H. Chace *et al.*, *Clin. Chem.* **49**, 1797 (2003).
5. M. G. Wilson, G. Jungner, “Principles and practice of screening for disease” (Public Health papers 34, World Health Organization, Geneva, 1968).
6. B. Wilcken, *Pathology* **44**, 73 (2012).
7. P. M. Farrell *et al.*, *J. Pediatr.* **147** (Suppl.), S30 (2005).
8. K. O. McKay, *J. Inherit. Metab. Dis.* **30**, 544 (2007).
9. S. D. Grosse *et al.*, *MMWR Recomm. Rep.* **53** (RR-13), 1 (2004).
10. M. S. Watson *et al.*, *Pediatrics* **117** (Suppl. 3), S296 (2006).
11. B. Wilcken *et al.*, *Pediatrics* **124**, e241 (2009).
12. L. F. Ross, *J. Inherit. Metab. Dis.* **35**, 627 (2012).
13. R. J. Pollitt, *J. Inherit. Metab. Dis.* **30**, 423 (2007).
14. HHS, Discretionary Advisory Committee on Heritable Disorders in Newborns and Children, Nominate a condition; www.hrsa.gov/advisorycommittees/mchbadvisory/heritabledisorders/.
15. R. H. Dees, J. M. Kwon, *Public Health Ethics* **6**, 114 (2013).
16. P. K. Duffner *et al.*, *Genet. Med.* **11**, 450 (2009).
17. J. R. Bonham, “Expanded Newborn Screening” (National Health Service, U.K. National Institute for Health Research, Sheffield Children’s Hospitals NHS Foundation Trust, 2012). www.expandedscreening.org/site/home/start.asp.
18. J. Heringer *et al.*, *Ann. Neurol.* **68**, 743 (2010).
19. A. M. Comeau, R. B. Eaton, *Science* **295**, 44 (2002).

Acknowledgments: The author thanks colleagues V. Wiley, K. Carpenter, and K. Bhattacharya.

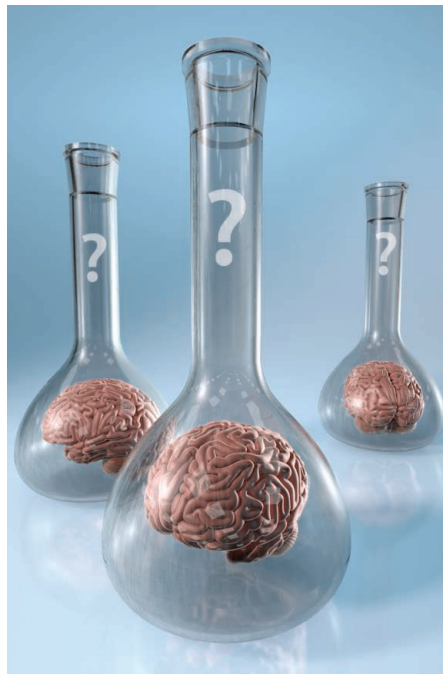
10.1126/science.1243944

What Are Mini-Brains?

Byoung-il Bae and Christopher A. Walsh

The human cerebral cortex defines us as who we are. Its development and function underlie complex human cognitive behavior, while its malfunction or degeneration causes countless neurological and psychiatric diseases. It has evolved markedly in humans compared to other animals and, therefore, no animal model truly recapitulates these human-specific features (1). We are currently limited to identifying genetic causes of abnormal brain development and function, observing brain shape and activity through imaging, and examining postmortem brain tissues. You simply cannot analyze human brain development directly. The more we try to model human disease in the mouse—with its miniscule cerebral cortex one-thousandth the size of a human's—the more we recognize the limitations of animal models. Lancaster *et al.* (2) have provided a major leap by developing a method to grow miniature human brainlike structures (cerebral organoids) from embryonic stem cells in vitro (2). The “mini-brains” recapitulate a surprising number of features of human embryonic brain development, heralding a new phase of modeling human disease.

Human and mouse embryonic stem (ES) cells, when cultured in special conditions, can produce organoids with a strong resemblance to precursors of the eyes, pituitary gland, and other brain structures (3). Remarkably, these organoids self-organize and display key features of the target organs, suggesting that many aspects of nervous system development are intrinsic to populations of stem cells and can be activated under the right growth conditions, even in the absence of many growth factors that had been thought to be essential. Lancaster *et al.* built upon this idea, developing a culture system for human ES cell-derived three-dimensional cerebral cortical organoids. This culture system introduces ES cells embedded in droplets of Matrigel (a gelatinous protein mixture) within a spinning bioreactor, providing the structural support and the nutrient/oxygen exchange that allows



Homegrown. A potential application for generating human cerebral organoids (brainlike structures) will be the ability to study brain development, model disease, and gain a better understanding of actual human physiology.

growth of larger, more complex organoids (up to 4 mm in diameter). Most growth factors are omitted from the medium, but retinoic acid, which is critical for cortical neurogenesis (4), is added to expedite development. In only 8 to 10 days, neurons appear, and in just 20 to 30 days, defined brain regions form. It takes months for this to happen in an actual human embryo.

Although a cerebral organoid is far smaller than the brain of an early fetus, it recapitulates characteristics of normal brain development, giving rise to a wide range of discrete brain regions, including forebrain, midbrain, hindbrain, meninges, choroid plexus, hippocampus, and retina. The cerebral cortex region is further subdivided, at least crudely, in a fashion analogous to division of the normal human brain into early motor, visual, and other areas. These regions seem to be interdependent, as markers of forebrain, for example, are adjacent to markers of hindbrain, suggesting mutual repression between regions.

Even regions within a cerebral organoid show the normal process of neocortical

Human cerebral organoids grown in the lab may quickly advance our understanding of brain development and disease.

development intriguingly well. For example, the horizontal, oblique, and vertical orientations of dividing stem cell progenitors closely resemble the trend in the human brain rather than in the mouse brain. Specialized progenitor cell types of the normal developing human brain also are recognizable, including outer radial glial (oRG) progenitors (1). Like the developing human brain, a cerebral organoid contains abundant oRG progenitors, whereas the developing mouse brain and cerebral organoid do not. Neurons in the organoids also show some (albeit not all) of the migration seen in a normal brain: Organoid neurons form both a “preplate” scaffold that regulates later-migrating neurons, and an intermediate, cell-sparse zone through which neurons migrate from deep in the brain to more superficial regions. As neurons assemble in the organoid cortex, they do so in a stereotypical manner that is considerably rougher than, but surprisingly similar to, what is seen in a normal brain. Interneurons migrate especially long distances from outside the cortex in vivo, and a similar type of migration is observed in organoids. As neurons differentiate in the organoids, they exhibit spontaneous Ca^{2+} surges or “action potentials,” which are the hallmark of functioning neurons in the brain.

Lancaster *et al.* further show that human cerebral organoids model some human diseases better than do mice. For example, the authors model microcephaly (“small brain”), which is caused by mutations in the gene *CDK5RAP2*. *CDK5RAP2* encodes a centrosomal protein that controls centriole replication (and therefore cell signaling and proliferation). Unlike humans, mice with *Cdk5rap2* mutations show only mild brain defects or more severe defects, depending upon the mouse strain (5, 6). When induced pluripotent stem (iPS) cells derived from skin fibroblasts from a microcephaly patient (with an aberrant *CDK5RAP2* gene) were cultured, cerebral organoids resembled human microcephaly—they were far smaller compared to control organoids due to premature neuronal differentiation at the expense of progenitor proliferation.

It is astonishing that human cerebral organoids can generate multiple distinct brain parts and functional cortical neurons

Division of Genetics and Genomics, Manton Center for Orphan Disease, and Howard Hughes Medical Institute, Children's Hospital Boston, Broad Institute of MIT and Harvard, and Departments of Neurology and Pediatrics, Harvard Medical School, Boston, MA 02115, USA. E-mail: christopher.walsh@childrens.harvard.edu

CREDIT: C. BICKELSCIENCE

intrinsically in vitro in such a short period of time, and display normal human cerebral cortex development broadly, if not perfectly. They do not grow beyond a 4-mm-diameter size, apparently because the lack of a blood supply limits access to nutrients. They lack many brain parts and cell types. And it is not yet clear how close the electrical potentials in organoids are to brain potentials, nor whether organoid neurons connect with the regions seen in an actual brain. Ethicists need not worry just yet, and may never need to worry, about the philosophical implications of “consciousness in a dish.” Indeed, perhaps the more interesting philosophical implication of these organoids is the extent to which these seemingly bland and undifferentiated (albeit totipotent) ES cells can

self-assemble into such a complex emergent structure.

As tissue engineering further improves the structure and reproducibility of these organoids, they will likely find their strongest application in the modeling of diseases (7). Combining new stem cell technologies with genome-editing tools, such as TALEN and CRISPR-Cas9 (8), will allow genetic modeling of many neurological and neuropsychiatric disorders. This may allow rapid screening of disease phenotypes, pathogenic mechanisms, and drug effects. Functional studies of human-specific genetic changes using human cerebral organoids may also be possible, providing insight into similar genes that act differently in humans and other mammals throughout evolution. Human pluripotent

ES cells and cerebral organoids promise to advance our understanding of neuroscience and stem cell biology ... and quickly.

Reference and Notes

1. J. H. Lui, D. V. Hansen, A. R. Kriegstein, *Cell* **146**, 18 (2011).
2. M. A. Lancaster *et al.*, *Nature* **501**, 373 (2013).
3. Y. Sasai, *Nature* **493**, 318 (2013).
4. J. A. Siegenthaler *et al.*, *Cell* **139**, 597 (2009).
5. S. B. Lizarraza *et al.*, *Development* **137**, 1907 (2010).
6. J. A. Barrera *et al.*, *Dev. Cell* **18**, 913 (2010).
7. Y. Sasai, *Cell Stem Cell* **12**, 520 (2013).
8. T. Gaj, C. A. Gersbach, C. F. Barbas 3rd, *Trends Biotechnol.* **31**, 397 (2013).

Acknowledgments: We thank M. A. Lancaster for discussion on the experimental details; W. F. Hu, M. B. Woodworth, and D. Jayaraman for critical reading; and the Walsh lab for general discussion.

10.1126/science.1245812

MATERIALS SCIENCE

Structure and Motion of a 2D Glass

Markus Heyde

Silicon oxide (silica) glass plays a key role in many modern technologies, from semiconductor devices and optical fibers to supporting materials in heterogeneous catalysis and novel durable glasses. Yet little is known about the atomic structure of amorphous materials. Recent studies of two-layer glass structures have started to shed light on the structure of amorphous silica

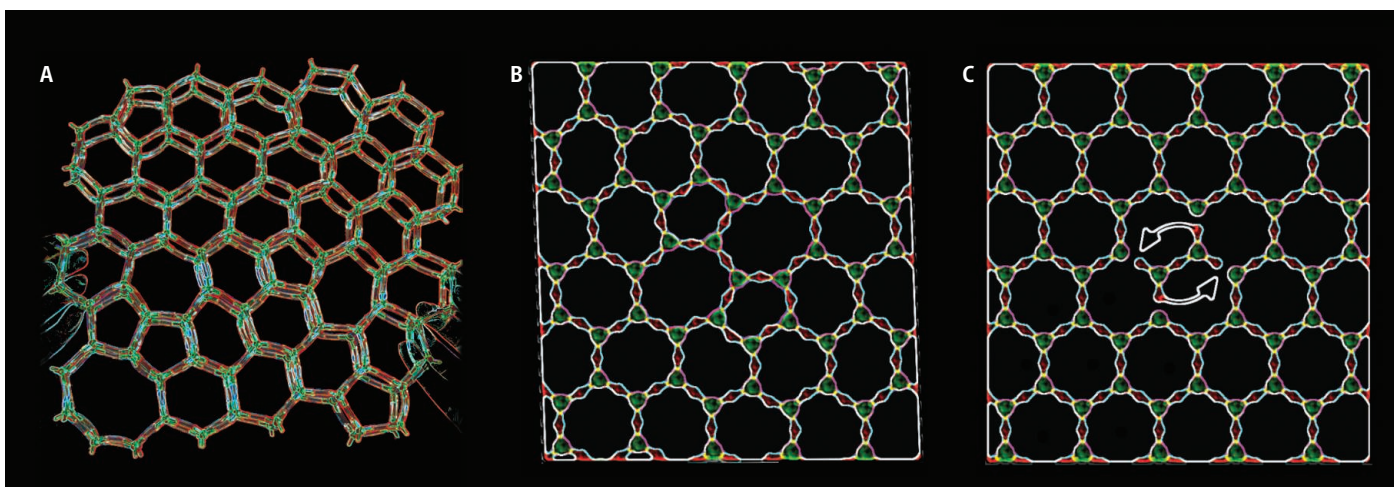
(1, 2). On page 224 of this issue, Huang *et al.* provide direct evidence for dynamic rearrangements of such two-dimensional (2D) silica films under a probing electron beam (3).

Diffraction methods are widely used to determine the structures of crystals and their surfaces. However, diffraction is of limited value for analyzing amorphous materials, which have no long-range order and periodicity. Zallen once wrote that “the atomic structure of an amorphous solid is one of its key mysteries, and structural information must be won with great effort” (4).

The structure and dynamics of a two-dimensional silica film provide fundamental insights into amorphous materials.

Transmission electron microscopy (TEM) and scanning tunneling microscopy (STM) methods have the potential to overcome these difficulties. In TEM, increased resolution as a result of aberration correction has brought a renaissance to the field (5). Scanning probe microscopes are now also capable of true atomic resolution. Molecular motions or even chemical reactions can be followed with both techniques (6–8). It has been proposed that noncrystalline materials can also be characterized with these methods (9, 10), but this has not yet been realized.

Fritz Haber Institute of the Max Planck Society, Faradayweg 4-6, 14195 Berlin, Germany. E-mail: heyde@fhi-berlin.mpg.de



Follow the motion. Silica consists of silicon and bridging oxygen atoms. In the 2D structure, crystalline silica has only six-membered rings. Zachariasen proposed more than 80 years ago that amorphous silica forms a network of rings

of different sizes (12); recent studies of 2D silica bilayers have verified this model (A) (1, 2). Huang *et al.* now provide experimental evidence for a transformation from amorphous (B) to crystalline structures (C) in such bilayers.

It is only as a result of the 2D flatness and defined structure of a recently developed class of materials that the characterization of amorphous solids at the atomic scale has become possible. Freund initiated the synthesis of thin films of silica and other oxides (11). The sample system that offers the clearest insights into amorphous systems is the bilayer silica film. The atomic structure of these bilayers can be tuned between crystalline and vitreous phases. Studies of such films have finally verified the atomic glass network, also known as random network theory, postulated by Zachariasen (12) more than 80 years ago. The film structure resembles the original 2D drawings in all of its atomic details (see the figure, panel A).

Recent studies with STM (1), followed by TEM (2), have revealed the atomic structure of amorphous silica bilayer films. The characterization of real-space data allows for a clear assignment of atomic sites. The position of oxygen and silicon atoms can be determined, and the ring structures, and their distribution and local neighborhood can be directly visualized. Chemical sensitivity imaging with STM and atomic force microscopy has

allowed direct assignment of all atomic species on the surface (13). Furthermore, the structural transition from a crystalline to an amorphous domain has been investigated by STM imaging of an interface region (14).

Huang *et al.* now report the observation of structural rearrangements in an amorphous silica bilayer film. The authors used a probing electron beam to deliberately cause these rearrangements. Remarkable images and videos show the movements of structural building blocks at the atomic scale. The opening and closing of ring structures and the subsequent rearrangements can be directly observed. The results open new ground for modeling the atomic structure and dynamics in glasses. By providing the opportunity to study vitreous materials at the atomic level, this unique model system is likely to have great impact on the general understanding of dynamic processes in amorphous bulk materials.

Future work might allow a direct assessment of atomic structures at the transition temperatures, where the liquid solidifies to either the crystalline or the amorphous state. Doping, adsorption, growth, and chemical reactivity studies of 2D glasses are another

focus of ongoing experiments. Band structure measurements or other material properties of 2D silica films might reveal unexpected features similar to those of graphene. Finally, 2D silica films can be grown on various substrates. Such films may find applications as new gate materials in the semiconductor industry.

References

1. L. Lichtenstein *et al.*, *Angew. Chem. Int. Ed.* **51**, 404 (2012).
2. P. Y. Huang *et al.*, *Nano Lett.* **12**, 1081 (2012).
3. P. Y. Huang *et al.*, *Science* **342**, 224 (2013).
4. R. Zallen, *The Physics of Amorphous Solids* (Wiley, New York, 1983).
5. H. Rose, *Optik* **85**, 19 (1990).
6. E. Nakamura, *Angew. Chem. Int. Ed.* **52**, 236 (2013).
7. J. Wintterlin, S. Völkening, T. V. W. Janssens, T. Zambelli, G. Ertl, *Science* **278**, 1931 (1997).
8. D. G. de Oteyza *et al.*, *Science* **340**, 1434 (2013).
9. R. Wiesendanger *et al.*, *Surf. Sci.* **181**, 46 (1987).
10. W. Raberg, V. Lansmann, M. Jansen, K. Wandelt, *Angew. Chem. Int. Ed. Engl.* **36**, 2646 (1997).
11. T. Schroeder *et al.*, *Surf. Rev. Lett.* **7**, 7 (2000).
12. W. H. Zachariasen, *J. Am. Chem. Soc.* **54**, 3841 (1932).
13. L. Lichtenstein, M. Heyde, H.-J. Freund, *J. Phys. Chem. C* **116**, 20426 (2012).
14. L. Lichtenstein, M. Heyde, H.-J. Freund, *Phys. Rev. Lett.* **109**, 106101 (2012).

10.1126/science.1245217

APPLIED PHYSICS

Directing Data Center Traffic

Yeshaiahu Fainman¹ and George Porter²

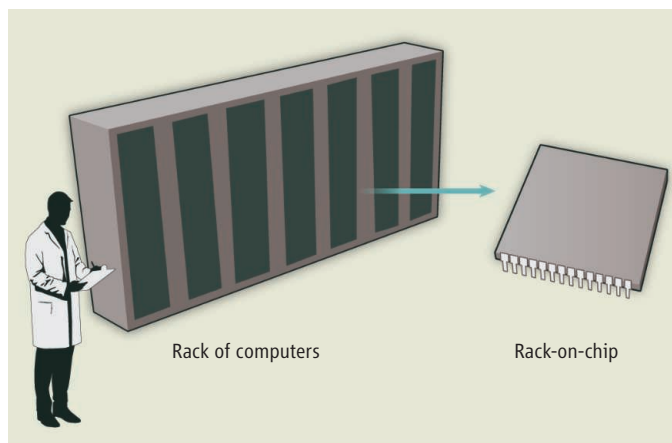
The widespread adoption of cloud computing has led to the construction of large-scale data centers hosting applications serving millions of users. Underpinning these data centers are tens to hundreds of thousands of servers that communicate internally with each other at high server-to-server bandwidths that are orders of magnitude greater than their connections to end users. Today's data centers consist of racks of 20 to 40 discrete servers, each configured with 8 to 16 CPU cores, hundreds of gigabytes of memory, and potentially tens of terabytes of storage. To meet cost and energy scaling requirements,

a new data center design will be required in which a rack of multiple, discrete servers, including the top-of-rack network switch, is integrated into a single chip (see the figure). These integrated "rack-on-chips" will be networked, internally and externally, with both

optical circuit switching (to support large flows of data), and electronic packet switching (to support high-priority data flows).

Numerous technological advances must be made for this vision to be realized. First, the energy efficiency of the processor cores must be improved to facilitate efficient heat dissipation, and this problem is the focus of many researchers in the field. We will focus instead on supporting better intra- and interprocessor networking. Although industrial efforts are under way to densely integrate optical networks within multicore processors (1), we argue that integrating rack-level networking requires more aggressive technology advancements.

Historically, optical technologies have enabled a large number of advancements in networking and communications, leading to the existence of the Internet with long-distance data transmission



Shrinking data centers. Evolution of a data center design in which a rack of multiple, discrete servers, including the top-of-rack network switch, is integrated into a single chip.

¹Department of Electrical and Computer Engineering, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA. ²Department of Computer Science and Engineering, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA. E-mail: fainman@eng.ucsd.edu; gmporter@cs.ucsd.edu

driven by power- and time-efficient regeneration. Recently, integrating circuit switching with packet switching has been examined with the goal to create hybrid networks within individual data centers (2, 3), using optics to provide more efficient services for applications relying on large flows of data. The key insight is that by quickly reconfiguring optical paths, changes in traffic workloads can be supported. As we envision optical networking interconnecting tightly integrated rack-on-chip designs, providing both on- and off-chip connections, we need even faster optical reconfiguration and cost-effective integration to support the highly variable traffic flows between individual processors on the chip and among the chips. Next-generation data center designs built with rack-on-chip designs will need to support both circuit and packet switching.

Each processor in the rack-on-chip design must have a transceiver, consisting of a transmitter and receiver. Each processor core would be interconnected with the other cores through an optical circuit switch, which allows communication paths to be set up and reconfigured between the cores (similar to the top-of-rack switch in current data centers). The high bandwidth between processor cores will require using both spatial and spectral (wavelengths) degrees of freedom, with wavelength-division multiplexing. Pairing each processor core with a transceiver requires miniaturizing transceivers and integrating them with the rack-on-chip design. The transceiver should be low-power and highly efficient, meaning that excessive heat is not generated, and that only a small number of photons over relatively short distances should be necessary to represent a bit of information, transmitted with low loss, and detected with an efficient receiver.

Nanophotonic technologies may meet the requirements for miniaturizing transceivers and circuit switches, using metamaterials, resonant nanostructures, nanoscale lasers, modulators, and receivers and switches. For example, it is possible to use advanced lithography to write and assemble devices and circuits into subsystems that support the rack-on-chip design. Recent advances in silicon photonics using complementary metal-oxide semiconductor-compatible manufacturing processes (4) mean that chip-scale, highly integrated optoelectronic solutions can be realized at low cost while meeting the other needs of scalability, bandwidth, fault tolerance, and energy efficiency. However, the efficient generation of light on a silicon chip is still in its infancy, and may not be able to overcome the fundamental issues prohibiting efficient generation

of light in indirect band-gap semiconductors. Alternative solutions similar to the delivery of electrical power from off-chip sources will bring the optical fields into the rack-on-chip. Or photons can be generated on the chip—for example, through heterogeneous integration of III-V compound semiconductor devices, such as nanolasers (5, 6).

To achieve highly scalable optical circuit architectures that can support many processor cores, each switching element must be miniaturized, relying on high optical nonlinearities that are very difficult to achieve in natural materials. One approach could be to develop nonlinear metamaterials, which are deeply subwavelength composites, engineered on atomic scale and/or a scale of a few atomic layers, exhibiting a qualitatively different response to radiation than that predicted by the effective medium theories of classical physics (e.g., crystal symmetry breaking, exotic semimetal modulation materials, and composite metal-dielectric nonlinear materials). The high nonlinear coefficient of new nonlinear metamaterials together with small-volume localized modes will enable low energy-per-bit operation.

Once this optical networking technology is integrated with electronic proces-

sors as a rack-on-chip design, the number of such chips can then be scaled up to meet the needs of future data centers. This will enable delivering new kinds of applications, such as computational climate modeling and biological applications, big data applications harnessing huge data sets, and online applications delivered to hundreds of millions of users.

References and Notes

1. M. Asghari, A. Krishnamoorthy, *Nat. Photon.* **5**, 268–270 (2011).
2. N. Farrington *et al.*, in *Proceedings of the ACM 2010 SIGCOMM Conference*, New Delhi, India, 30 August to 3 September 2010 (Association for Computing Machinery, New York, 2010), pp. 339–350.
3. G. Wang *et al.*, in *Proceedings of the ACM 2010 SIGCOMM Conference*, New Delhi, India, 30 August to 3 September 2010 (Association for Computing Machinery, New York, 2010), pp. 327–338.
4. "Luxtera, Freescale collaborate on CMOS photonics fab," <http://optics.org/article/39426>, 9 June 2009.
5. M. P. Nezhad *et al.*, *Nat. Photon.* **4**, 395–399 (2010).
6. M. Khajavikhan *et al.*, *Nature* **482**, 204–207 (2012).

Acknowledgments: Supported by the Google Focused Research Award, the Defense Advanced Research Projects Agency, NSF, NSF Engineering Research Center for Integrated Access Networks, the Office of Naval Research, the Cymer Corporation, the U.S. Army Research Office, and the UCSD Center for Networked Systems.

10.1126/science.1242906

DEVELOPMENT

Getting Your Gut into Shape

Benjamin D. Simons^{1,2,3}

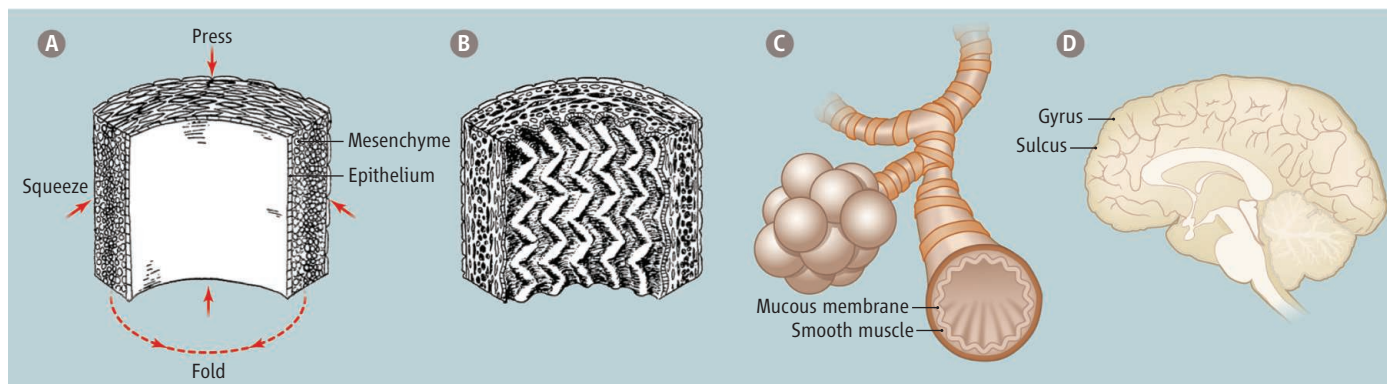
Mechanical forces exerted between tissue layers in the intestine are all that is needed to make gut villi.

The specification and patterning of plant and animal tissues relies upon the spatial and temporal coordination of biochemical and physical processes at the molecular, cellular, and tissue scale (1, 2). Yet, despite access to genetic manipulation techniques and in vivo live-imaging platforms, progress in understanding how these processes interact in development has proved challenging. Reliant on the interplay of gene regulatory and mechanical cues, the emergence of spatial organization in the gut epithelium provides a paradigm for morpho-

genic processes in vertebrates. On page 212 of this issue, Shyer *et al.* (3) combine in vitro analyses of tissue explants with the development of a biophysical modeling scheme to show that the seemingly complex process of intestinal villi specification can be explained simply through the action of mechanical constraints.

In vertebrates, the digestive tract arises from a primitive gut tube (4). As the gut matures, the foregut, midgut, and hindgut become morphologically distinct, before differentiating into specialized primary organs: The foregut (pharynx, esophagus, and stomach) is responsible for ingestion and the initiation of digestion, whereas the midgut (small intestine) provides the major site of digestion and nutrient absorption, and the hindgut (large intestine) resorbs water and expels waste. To fulfill these functions, the

¹Cavendish Laboratory, Department of Physics, J. J. Thomson Avenue, University of Cambridge, Cambridge CB3 0HE, UK. ²The Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QN, UK. ³Wellcome Trust-Medical Research Council Stem Cell Institute, University of Cambridge, UK. E-mail: bds10@cam.ac.uk



gut organ epithelia acquire distinct luminal morphologies (5). In particular, to maximize the absorptive area of the epithelium, the mucosa of the small intestine is remodeled into an intricate array of finger or leaf-like protrusions, known as villi, which extend into the lumen. At the base of the villi, smaller glands, known as crypts of Lieberkühn, form invaginations that open onto the luminal surface and provide a niche environment for the stem cells that support the rapid turnover of the epithelium (6).

For many organisms, the morphology of the small intestine is not specified directly through a genetically determined developmental program but instead emerges in a sequence of morphological alterations (5, 7). This stepwise progression is exemplified by the chick: Early in development, the small intestine comprises a smooth cylinder of mesenchyme, supported by an external layer of mesothelium and an internal layer of epithelium (see the figure, panel A). As the epithelium expands by cell division and the mesenchyme differentiates to form a layer of circularly oriented smooth muscle, a regular array of “previllous” ridgelike structures develops along the length of the gut. Later, two further layers of longitudinal smooth muscle appear sequentially on either side of the circular muscle, the first accompanied by a transition of the ridges into a herringbone pattern of zigzags (see the figure, panel B) and the second leading to the emergence of villi. Finally, late in development, crypts form following the specification of progenitors at the base of the villi, which form invaginations into the submucosa.

Previous studies have conjectured that mechanical factors might drive morphogenesis in the intestine (5, 7, 8). However, the question of whether patterning emerges “passively” from the resolution of mechanical stresses imposed by the expansion of the epithelium against the muscle layer, or whether it results from the active contraction of individual cells, remained open. By designing a combination of in vitro assays, Shyer *et al.* show

that the emergence of previllous structures in the chick embryo relies on mechanical constraints imposed by muscle differentiation. As well as demonstrating that the separation of the muscle layer from the internal mesenchyme and epithelium inhibits the formation of ridgelike structures in vitro, they show that patterning can be restored simply by constraining the circumferential expansion of the muscle-free gut using a porous silk tube. Similarly, they have shown that the subsequent “buckling” of the ridges can be abolished by chemically inhibiting the first round of longitudinal muscle differentiation, whereas inhibition of the second round prevents transformation of the zigzag pattern into villi.

To further elucidate the dynamic process of remodeling, Shyer *et al.* develop a minimal biophysical modeling scheme, based only on the minimization of the strain energy of the layered structure, treating the growing tissue as a geometrically constrained elastic material. Within this scheme, they provide a quantitative basis for the origin and scale of the sequence of alterations and, through adjustments in the constitutive parameters, account for the diverse range of villi structures seen in different organisms, from *Xenopus* and zebrafish to snakes and mice.

Numerous studies have emphasized the importance of differential growth and mechanical stress in shaping the morphology of multicellular organisms and tissues (9–11). Patterning in airways (12), arteries (13), skin (14), and brain (15) have all been associated with buckling-like instabilities created by mechanical forces acting on a growing tissue (see the figure, panels C and D). The present study provides a firm experimental basis for mechanics not just contributing to, but driving, patterning in the intestinal epithelium.

In the light of this work, it is natural to question whether the final stage of intestinal patterning, the invagination of crypts, can also be accounted for by mechanical forces. However, the remarkable ability of individual intestinal stem cells to reconstitute organized

Tissue “origami” builds gut villi. Expansion of the growing epithelium in the small intestine of vertebrates against layers of muscle in the mesenchyme (A) leads to buckling and the progressive emergence of patterned fingerlike structures known as villi (B) [after (7)]. A similar buckling mechanism is thought to account for the appearance of textures in a variety of layered tissues from (C) the folds in the mucosal lining of the bronchioles in lung to (D) the convoluted structures of the cerebral cortex in large mammals.

cryptlike structures in vitro, in the absence of an underlying mesenchyme (16), points to a different mechanism where transcriptional regulation and cell-cell interactions conspire with mechanical cues to engineer a stereotyped cellular organization.

In the postgenomic era, it has become anathema to speak about “mechanism” without reference to interactions of genes and gene products. The current study is instructive in that, without addressing information on subcellular processes, it fulfills the objective of mechanistic understanding, providing an explanation of the emergence of patterning and offering predictive new insights.

References

1. C. T. L. Wolpert, *Principles of Development* (Oxford Univ. Press, Oxford, 4th ed., 2011).
2. C.-P. Heisenberg, Y. Bellaïche, *Cell* **153**, 948 (2013).
3. A. E. Shyer *et al.*, *Science* **342**, 212 (2013).
4. A. M. Zorn, J. M. Wells, *Annu. Rev. Cell Dev. Biol.* **25**, 221 (2009).
5. W. A. Hilton, *Am. J. Physiol.* **1**, 459 (1902).
6. H. Clevers, *Cell* **154**, 274 (2013).
7. A. J. Coulombre, J. L. Coulombre, *J. Embryol. Exp. Morphol.* **6**, 403 (1958).
8. D. R. Burgess, *J. Embryol. Exp. Morphol.* **34**, 723 (1975).
9. D. W. Thompson, *On Growth and Form* (Cambridge Univ. Press, Cambridge, 1942).
10. L. A. Taber, *Appl. Mech. Rev.* **48**, 487 (1995).
11. J. Dervaux, M. Ben Amar, *J. Mech. Phys. Solids* **59**, 538 (2011).
12. R. K. Lambert *et al.*, *J. Appl. Physiol.* **77**, 1206 (1994).
13. A. Goriely, R. Vandiver, *IMA J. Appl. Math.* **75**, 549 (2010).
14. M. Kücken, A. C. Newell, *J. Theor. Biol.* **235**, 71 (2005).
15. D. P. Richman *et al.*, *Science* **189**, 18 (1975).
16. T. Sato, H. Clevers, *Science* **340**, 1190 (2013).

10.1126/science.1245288

ECOLOGY

Cleaner Lakes Are Dirtier Lakes

Emily S. Bernhardt

Phosphorus pollution can often explain the difference between a lake clogged with algae and a clear lake where people flock to fish and swim. Although nutrient pollution remains the most common source of water-body impairment throughout much of the developed world (1), phosphorus management has reduced algal blooms in many temperate lakes worldwide (2). Yet on page 247 of this issue, Finlay *et al.* (3) report a downside to this success. In a series of lakes in which phosphorus inputs have declined, they show substantial increases in nitrate pollution.

To understand this phenomenon, we must first recognize that nitrogen and phosphorus cycles in lakes are linked. Algae require 10 to 40 times as much nitrogen as phosphorus (4), but phosphorus is typically in much shorter supply than nitrogen, and the growth of algae in most temperate lakes is therefore phosphorus-limited. It tends to remain that way because whenever phosphorus loading to lakes increases, nitrogen-fixing blue-green algae ensure that there is sufficient nitrogen by converting atmospheric dinitrogen (N_2) into tissue nitrogen (5).

Finlay *et al.* suggest that historic phosphorus pollution led to large amounts of pollutant nitrogen being assimilated into the tissues of algal blooms, which were exported to the lake bottom as those algae died (see the figure, panel A). In high-phos-

phorus lakes, algal blooms efficiently move large amounts of nitrogen and phosphorus together with recently fixed algal carbon into the subsurface, fueling high microbial activity and oxygen consumption. The high supply of carbon, together with low oxygen, provides ideal conditions for denitrification, the microbial metabolic pathway in which microbes breathe nitrate (NO_3) while decomposing organic substrates, converting the pollutant NO_3 to the inert N_2 gas that makes up 78% of our atmosphere.

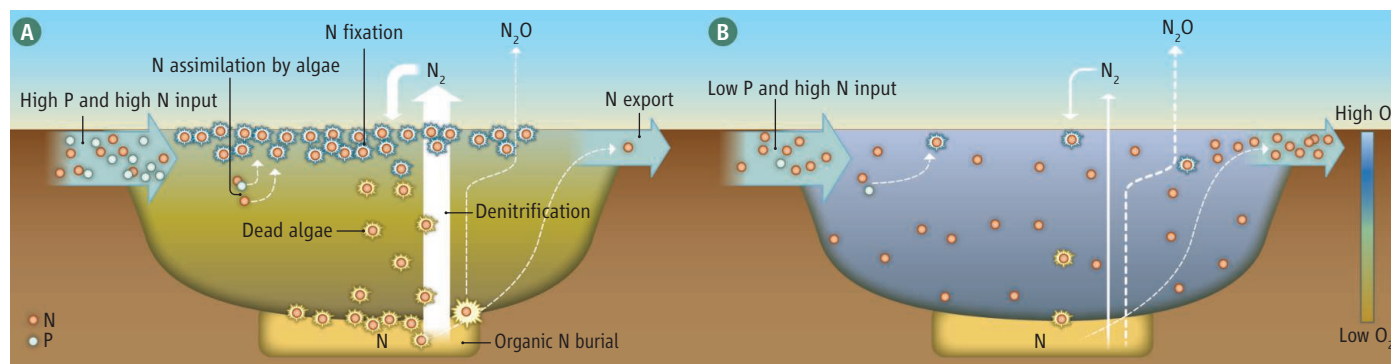
As phosphorus supply to lakes declines, there is no mechanism equivalent to nitrogen fixation by which algae can generate additional phosphorus. Excess nitrogen thus remains unused in lake water. Denitrifiers are an important sink for nitrogen in all lakes, but their ability to remove nitrogen is constrained by the supply of energy (in the form of organic matter) and requires the absence of oxygen. Reduced algal biomass due to reduced phosphorus loading means that less organic matter falls to a lake's depths, oxygen supplies are not depleted, and microbial denitrification is not energetically favorable (see the figure, panel B).

These considerations explain why good-faith efforts to manage phosphorus appear to be leading to lakes that are clearer and better oxygenated but more polluted with nitrogen. This nitrogen is exported to downstream waters, ultimately contributing to pollution problems in coastal waters, where algae are typically limited by nitrogen. Lakes are important nitrogen sinks (6, 7), and the lakes

Efforts to reduce the phosphorus input to lakes have helped to lessen algal blooms but exacerbated nitrate pollution.

in Finlay *et al.*'s synthesis are no exception, removing up to 90% of nitrogen inputs. Yet their results raise concerns that this nitrogen retention capacity is declining as a consequence of phosphorus load reductions. Recent efforts to model the movement of nitrogen across the continental United States suggest that nitrogen retention in lakes and reservoirs is critically important in reducing the amount of terrestrial nitrogen runoff that makes its way into coastal oceans (8). Finlay *et al.*'s synthesis suggests that some part of this historic nitrogen retention efficiency in lakes should be attributed to phosphorus pollution.

Regulations intended to improve air and water quality typically focus on one problem at a time, ignoring the reality that pollutants interact. For example, clean air legislation required the installation of smokestack scrubbers to reduce acid rain. This intervention successfully reduced sulfur dioxide emissions, but the acidity of rain was not as strongly affected as anticipated because scrubbers also reduced atmospheric concentrations of neutralizing dust (9). The same aerosol reductions also unexpectedly exacerbated global warming trends by allowing more sunlight to reach Earth's surface (10). Although these unanticipated consequences in no way change the correctness of the decision to reduce particulate matter, they do suggest that predicting the effects of new regulations requires consideration of the complex interactions between pollutants.



Lake pollution revisited. (A) High phosphorus loading to lakes leads to the assimilation of large amounts of both nitrogen (N) and phosphorus (P) by algal blooms. N fixation ensures that the algae can take advantage of all available P. Export of algal tissues from the surface to the lake bottom leads to the burial or denitrification of assimilated N. Under these conditions, little N is exported from the lake to down-

stream rivers. (B) The results reported by Finlay *et al.* demonstrate that as P inputs decline, algal growth and N assimilation also decline, and the biological pumping of N into lake sediments slows down. Without high rates of assimilating and sinking together with burial or denitrification, more nitrogen remains in the surface waters of these clearer lakes. This unused nitrogen is then exported downstream.

Finlay *et al.*'s work provides another example of such unintended consequences of solving only one of two linked environmental problems. Their analysis raises important questions about the spatial scale of nutrient management efforts. Phosphorus reductions have unquestionably improved conditions in many temperate lakes, but these improvements may exacerbate nitrogen pollution problems in sensitive coastal waters. Rising nitrate concentrations in fresh waters may also have implications for global climate, because nitrogen loading has been shown to enhance the

production of the potent greenhouse-warming and ozone-destroying gas nitrous oxide (N_2O) in lakes (11, 12), wetlands, and streams. It is high time to consider the substantial environmental benefits at all scales that will be gained by addressing these tightly linked, essential, and overused nutrients—nitrogen and phosphorus—simultaneously (13).

References

1. S. Carpenter *et al.*, *Ecol. Appl.* **8**, 559–568 (1998).
2. E. Jeppesen *et al.*, *Freshw. Biol.* **50**, 1747–1771 (2005).
3. J. C. Finlay, G. E. Small, R. W. Sterner, *Science* **342**, 247 (2013).

4. C. A. Klausmeier, E. Litchman, T. Daufresne, S. A. Levin, *Nature* **429**, 171–174 (2004).
5. V. H. Smith, *Science* **221**, 669–671 (1983).
6. M. Janssen, R. Andersson, H. Berggren, L. Leonardson, *Ambio* **23**, 320 (1994).
7. D. A. Bruesewitz, D. P. Hamilton, L. Schipper, *Ecosystems* **14**, 341–352 (2011).
8. J. Harrison *et al.*, *Biogeochem.* **93**, 143–157 (2009).
9. M. Wild, H. Gilgen *et al.*, *Science* **308**, 847–850 (2005).
10. L. O. Hedin, G. E. Likens, *Sci. Am.* **275**, 88–92 (1996).
11. M. L. McCrackin, J. J. Elser, *Global Biogeochem. Cycles* **25**, GB4005 (2011).
12. M. L. McCrackin, J. J. Elser, *Ecology* **91**, 528–539 (2010).
13. D. J. Conley *et al.*, *Science* **323**, 1014–1015 (2009).

10.1126/science.1245279

GENETICS

GWAS to Therapy by Genome Edits?

Ross C. Hardison¹ and Gerd A. Blobel^{2,3}

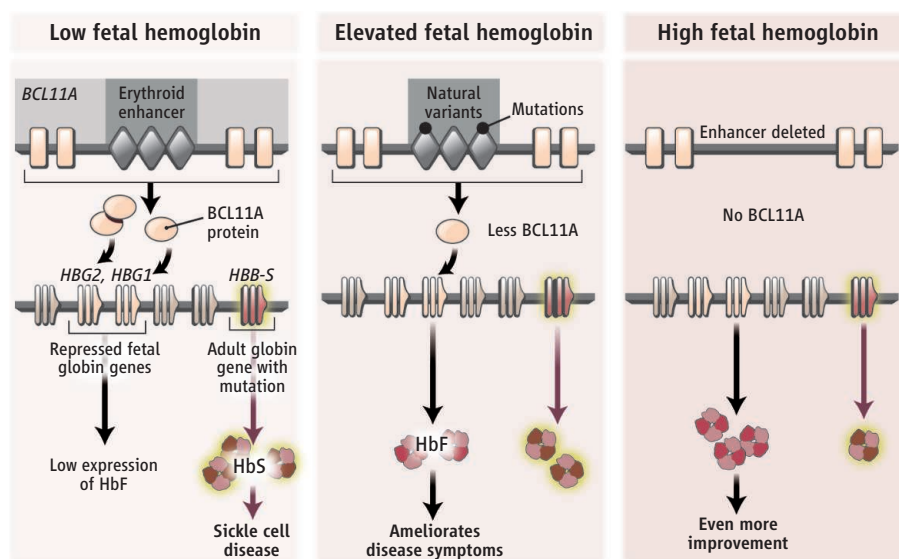
Disorders of hemoglobins are the most common monogenic diseases in the world, with substantial morbidity and mortality resulting from either defective function of the protein, such as in sickle cell anemia, or from insufficient protein production, such as the thalassemias (1). Genome-wide association studies (GWAS) have implicated two genes other than the globin genes as potential modulators of the pathology of these diseases by influencing the amounts of fetal hemoglobin (HbF). On page 253 in this issue, Bauer *et al.* (2) characterize common single-nucleotide polymorphisms (SNPs) in one of these genes, *BCL11A*. SNPs associated with mild increases in HbF amounts reside within a powerful tissue- and developmental stage-specific *BCL11A* enhancer (see the figure). Genome engineering reveals that this enhancer is essential for erythroid expression of *BCL11A*, and as a consequence, for globin gene expression. This exquisite specificity points to genome editing as a plausible approach to lasting corrective cell-specific therapy for certain hemoglobinopathies.

The first disease-linked mutation defined at the molecular level was the amino acid substitution in β -globin that leads to sickle cell anemia (3). Since then, genetic alterations have been identified as underlying causes for diverse congenital hemoglobinopathies. The search for therapies based on genetic

insights has driven investigation of the genetics and regulatory machinery of the globin genes. Most vertebrates produce different forms of hemoglobin during development, and humans produce a fetal stage-specific form called HbF. The concentration of HbF normally declines after birth, but the amount of HbF persisting in normal adults is a variable trait with strong heritability. Elevated HbF amounts can attenuate the symptoms in patients with sickle cell disease or thalassemias (4). GWAS initially identified two

Genetic and epigenetic studies of gene variants reveal a potential genomic target for treating hemoglobin disorders.

quantitative trait loci, not linked to the β -like globin genes, that determine the amount of HbF produced in normal populations (5, 6). One of these loci encodes *BCL11A*. Notably, ~15% of the variation in HbF in sickle cell anemia populations is accounted for by SNPs in *BCL11A*, which is high compared to most GWAS. *BCL11A* is a transcription factor that represses embryonic and fetal β -like globin transcription in human and mouse erythroid cells, and is a dominant regulator of developmental globin gene expression (7, 8). *Bcl11a*



Exploiting nature's variants. (Left) An erythroid enhancer promotes expression of *BCL11A*, which encodes a repressor that silences fetal globin genes *HBG1* and *HBG2* in adult human erythroid cells. The globin gene *HBB* is expressed in adult erythroid cells, but if mutated, disease can ensue, such as the production of sickle cell hemoglobin (HbS). (Middle) Natural variants in the enhancer reduce *BCL11A* production and boost fetal globin gene expression. Production of fetal hemoglobin (HbF) can ameliorate symptoms of some hemoglobinopathies. (Right) Removal of the enhancer is expected to reduce the amount of *BCL11A*, thereby allowing expression of the fetal globin genes and improving the pathologies from *HBB* mutations.

¹Department of Biochemistry and Molecular Biology, Center for Comparative Genomics and Bioinformatics, The Pennsylvania State University, University Park, PA 16802, USA. ²Division of Hematology, The Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA. ³Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA. E-mail: rch8@psu.edu; blobel@email.chop.edu

loss ameliorates sickle cell anemia in mouse models of the disease (9).

Bauer *et al.* show that a region within an intron of the *BCL11A* gene has epigenetic signatures indicative of a transcriptional enhancer, as reflected in hypersensitivity to deoxyribonuclease, histone modifications associated with active enhancers, and physical juxtaposition with the *BCL11A* promoter regions through chromatin looping. SNPs with the most pronounced effects on HbF concentration fall at or near these critical regulatory sequences, and in one case impairs binding of erythroid transcription factors. In transgenic mice, this enhancer is sufficient to drive expression of a reporter gene predominantly in the fetal liver, the initial site of adult-type erythropoiesis in the developing mouse. Its deletion from the *Bcl11a* gene in a murine erythroid cell line, but not in a lymphocyte cell line, impaired BCL11A production, accompanied by an increase in embryonic globin expression, thus confirming the erythroid specificity of the enhancer.

More broadly, the results of Bauer *et al.* inform our understanding and expectations of the role of regulatory variants in complex traits, including disease susceptibility. Trait-associated variants discovered in GWAS are enriched in DNA segments carrying epigenetic hallmarks of regulatory regions (10, 11). However, most variants have modest effects on the phenotype, as is the case for the SNPs at the *BCL11A* locus. Yet, examination of the genomic context of these trait-associated variants has led to the identification of an

enhancer with a powerful effect on expression of *BCL11A* and, indirectly, the globin genes. Thus, the modest phenotypic effects of the common variants mapped in GWAS need not be interpreted as indicative of modest effects of the regulatory region or locus in which they reside. Rather, they should be considered as components of a regulatory complex that in total could have strong effects on phenotype.

Gene replacement therapy for the hemoglobinopathies has proved to be an extraordinarily difficult prospect (12). An alternative strategy to raise HbF concentrations would be to reduce BCL11A amounts or activity. However, *BCL11A* is widely expressed, and its loss is lethal due to nonerythroid effects (13). Those considerations and the generally undruggable nature of transcription factors led to the initial view of BCL11A as an implausible target for therapeutic intervention. The study of Bauer *et al.*, however, raises the possibility of crippling the erythroid enhancer through genome editing in human hematopoietic stem or progenitor cells. Cells thus modified would be expected to display loss of *BCL11A* expression in erythroid cells while maintaining it in nonerythroid lineages. The potential benefits would be an increase in HbF production; a reciprocal decrease in expression of the defective adult globin in the case of sickle cell anemia; permanence of a one-time genetic deletion compared to gene replacement therapies that require long-term sustained expression of a transgene; and a selective growth advan-

tage of modified erythroid cells over unmodified counterparts.

Advances in genome editing (14, 15) are moving enhancer modifications into the arsenal of gene therapy approaches. However, additional work in animal models and primary human cells is needed to confirm and extend characterization of the enhancer, with emphasis on measuring effects on other tissues or genes. Further improvements in genome editing are essential to maximize efficiency and minimize off-target genomic alterations. Despite these challenges, the results of Bauer *et al.* suggest that genomic modification of an erythroid enhancer might ameliorate hemoglobinopathies, and the old dream of genetically guided therapies for this disease spectrum may become a reality.

References and Notes

1. T. N. Williams, D. J. Weatherall, *Cold Spring Harbor Perspect. Med.* **2**, a011692 (2012).
2. D. E. Bauer *et al.*, *Science* **342**, 253 (2013).
3. V. M. Ingram, *Nature* **178**, 792 (1956).
4. R. L. Nagel, *Semin. Hematol.* **28**, 180 (1991).
5. S. Menzel *et al.*, *Nat. Genet.* **39**, 1197 (2007).
6. M. Uda *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **105**, 1620 (2008).
7. V. G. Sankaran *et al.*, *Science* **322**, 1839 (2008).
8. V. G. Sankaran *et al.*, *Nature* **460**, 1093 (2009).
9. J. Xu *et al.*, *Science* **334**, 993 (2011).
10. The ENCODE Project Consortium, *Nature* **489**, 57 (2012).
11. M. T. Maurano *et al.*, *Science* **337**, 1190 (2012).
12. M. Cavazzana-Calvo *et al.*, *Nature* **467**, 318 (2010).
13. P. Liu *et al.*, *Nat. Immunol.* **4**, 525 (2003).
14. J. Boch *et al.*, *Science* **326**, 1509 (2009).
15. P. Mali *et al.*, *Science* **339**, 823 (2013).

Acknowledgments: We thank V. Sankaran for helpful comments. Supported by grants R37DK058044 and R01DK054937 (G.A.B.) and R01DK065806 and U54HG006998 (R.C.H.).

10.1126/science.1245813

MOLECULAR BIOLOGY

RNAi, Antiviral After All

Selena M. Sagan¹ and Peter Sarnow²

RNA interference (RNAi) is an evolutionarily conserved pathway in cells for potent and specific silencing of gene expression. It is triggered by the accumulation of double-stranded RNA (dsRNA), which is subsequently processed into small interfering RNA (siRNA) and taken up into a protein complex that silences genes by cleaving their complementary RNAs (1). This mechanism has an important function in immunity against viruses in infected plants and invertebrates, but whether this is true

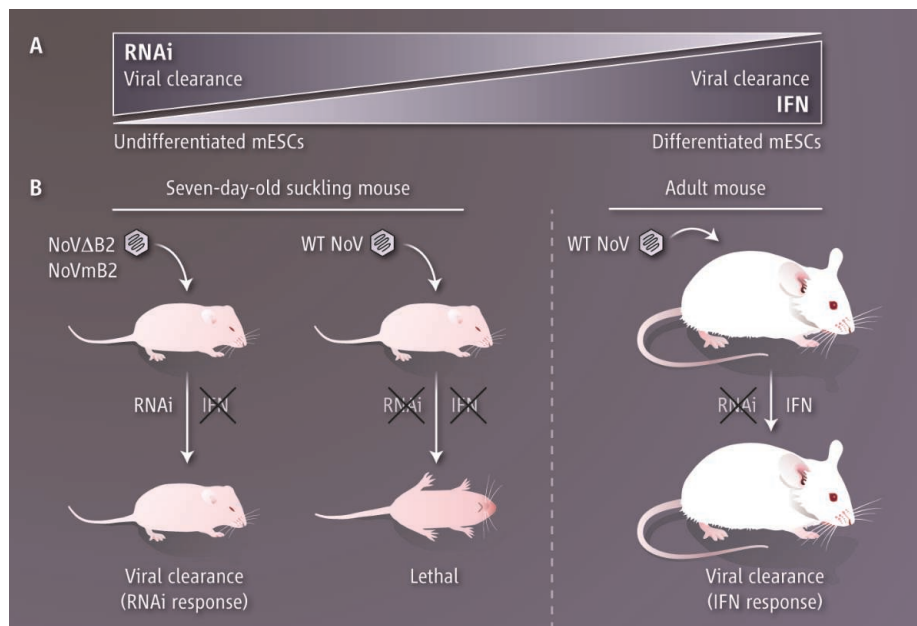
in mammals has been widely debated (2). On pages 235 and 231 of this issue, Maillard *et al.* (3) and Li *et al.* (4) provide evidence for the existence of a functional antiviral RNAi pathway in mammalian cells.

There has been a lack of compelling evidence for virus-derived small RNAs (vsRNAs) in mammalian cells (5). vsRNAs were detected in 41 human cell lines infected with six different RNA viruses, but it was unknown whether the vsRNAs were functional (6). It has been suggested that mammals have supplanted the RNA-based antiviral RNAi pathway with the protein-based, antiviral interferon (IFN) response (2, 7).

No longer elusive, mammalian antiviral RNA interference is now confirmed.

Maillard *et al.* noted that undifferentiated mouse embryonic stem cells (mESCs) lack an innate immune response that normally is stimulated in the presence of long dsRNA (2, 8). However, mESCs support the incorporation of siRNAs into functional RNA-induced silencing complexes (RISCs), demonstrating that mESCs contain an intact RNAi pathway (8–11). Thus, the authors reasoned that mESCs were a good experimental system to detect potential antiviral RNAi responses in the absence of innate IFN responses. Indeed, the authors observed that upon infection of mESCs with encephalomyocarditis virus (EMCV), vsRNAs were associated with the Argonaute 2 (the protein that loads RNA into

¹Department of Microbiology and Immunology, McGill University, Montreal, Quebec H3A 2B4, Canada. ²Department of Microbiology and Immunology, Stanford University, Stanford, CA 94305, USA. E-mail: psarnow@stanford.edu



Antiviral RNAi in mammals. (A) RNAi and the IFN response function at different times in mESCs. (B) Upon infection with an RNA virus (NoV) that lacks (NoVΔB2) or expresses a mutant (NoVmB2) viral RNAi suppressor, suckling mice mount an antiviral RNAi response that clears the infection. The lethality of wild-type (WT) NoV infections is likely due to viral suppression of antiviral RNAi and the lack of IFN responses at this early stage of mouse development. In adult mice, the IFN response supplants antiviral RNAi in viral clearance.

RISC) and were undetectable in cells lacking Dicer, the enzyme that cleaves dsRNAs into siRNAs. Accumulation of EMCV vsRNAs was reduced upon cell differentiation (see the figure), whereas the abundance of most siRNAs remained unchanged. This was attributed to the establishment of the IFN response, supplanting the RNAi antiviral response upon cell differentiation. However, the function of these EMCV vsRNAs was not clear.

A potential function for vsRNAs could be ascertained, however, from infection studies of mESCs with Nodamura virus (NoV). Wild-type NoV encodes a suppressor of RNAi called B2. This protein is essential for virus multiplication in that it inhibits Dicer (12). Maillard *et al.* found that wild-type NoV infected mESCs with high efficiency compared to NoV lacking B2 (NoVΔB2). Deep sequencing revealed virally derived RNAs with canonical features of 22-nucleotide siRNAs. Furthermore, replication of NoVΔB2 could be rescued by ectopic expression of either B2 or the Ebola virus VSR protein (VP-35), which suppresses mammalian Dicer through an alternative mechanism to B2 (13). These results indicate that NoVΔB2's growth defect is due to its lack of suppression of RNAi, which is normally a potent antiviral pathway in mESCs.

Li *et al.* investigated whether vsRNAs can be generated in infected animals. Although NoV infections were lethal to 7-day-old suckling mice, NoVΔB2 infections resulted in a factor of 1000 lower viral RNA abundance,

and the mice remained healthy for the duration of the experiments. Gene expression profiles of known innate antiviral pathways revealed no major differences between mice infected with either virus, suggesting that the rapid clearance of NoVΔB2 was not due to an innate immune response (4). A caveat of this interpretation is that certain genes of the innate response can have high constitutive expression. Furthermore, a mutant virus, NoVmB2, which contains a mutation in B2 protein that abolishes its RNAi suppressor function, was also nonvirulent in suckling mice. Deep sequencing revealed accumulation of vsRNAs with canonical features of 22-nucleotide siRNAs in mice infected with either mutant virus, but not with wild-type virus. Thus, without suppression of the RNAi system by the virus, suckling mice can mount a sufficiently potent antiviral RNAi response to protect against infection. By contrast, NoV-infected adult mice clear the virus with antiviral protein-based IFN responses.

The evidence for a functional antiviral RNAi pathway in mammals suggests that both RNA-based and protein-based antiviral mechanisms operate in mammalian tissues, possibly at the same time. Indeed, it is likely that RNAi-based and IFN-based antiviral immune responses intersect. For example, in vitro biochemical studies suggest that innate immune factor PACT can bind to both the dsRNA-activated protein kinase PKR, which is part of the protein-based response, and to

TRBP, which modulates Dicer activity in the RNA-based response (14, 15).

Why has mammalian antiviral RNAi been so elusive to date? As Maillard *et al.* and Li *et al.* point out, antiviral RNAi may have been overlooked in previous studies, most of which have invariably used viruses that may encode potent viral suppressors of RNAi. Furthermore, Li *et al.* note that most studies have been carried out in differentiated mammalian cells that can mount potent and rapid IFN responses. These findings raise the question as to whether some differentiated cell types, but not others, can mount antiviral RNAi responses. It is still unclear whether tissue-specific RNAi mechanisms could explain tissue tropism and pathogenic signatures of certain viruses in infected animal hosts. Also unknown is whether there exists an RNA-based response to viruses that do not elicit protein-based innate immune responses.

The finding that suckling mice retain an antiviral RNAi mechanism is intriguing. Whether this indicates that a subset of fetal cells has retained a diverse RNA-based antiviral system to combat viruses—and if so, when during development, and in which tissues the RNAi pathway is inhibited—should be explored. Seo *et al.* (16) noted that members of microRNA cluster miR-17/93 negatively regulate the expression of IFN-stimulated genes (ISGs). However, virus infection could inhibit RNAi activity by poly-ADP-ribosylation of RISC, thereby reducing miR-17/93 and increasing ISGs. In this scenario, RNAi does not play an antiviral role. A better understanding of the antiviral RNAi pathway will help to answer these questions and elucidate the intersection of the protein-based and RNA-based immune responses in mammalian development and evolution.

References

1. S. W. Ding, O. Voinnet, *Cell* **130**, 413 (2007).
2. J. L. Umbach, B. R. Cullen, *Genes Dev.* **23**, 1151 (2009).
3. P. V. Maillard *et al.*, *Science* **342**, 235 (2013).
4. Y. Li *et al.*, *Science* **342**, 231 (2013).
5. S. Pfeffer *et al.*, *Nat. Methods* **2**, 269 (2005).
6. P. Parameswaran *et al.*, *PLOS Pathog.* **6**, e1000764 (2010).
7. D. J. Obbard *et al.*, *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **364**, 99 (2009).
8. E. Billy *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 14428 (2001).
9. E. P. Murchison *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 12135 (2005).
10. H. Su *et al.*, *Genes Dev.* **23**, 304 (2009).
11. P. J. Paddison, A. A. Caudy, G. J. Hannon, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 1443 (2002).
12. C. S. Sullivan, D. Ganem, *J. Virol.* **79**, 7371 (2005).
13. G. Fabozzi *et al.*, *J. Virol.* **85**, 2512 (2011).
14. M. Singh *et al.*, *Biochemistry* **50**, 4550 (2011).
15. H. Y. Lee *et al.*, *Nucleic Acids Res.* **41**, 6568 (2013).
16. G. J. Seo *et al.*, *Cell Host Microbe* **10**, 1016 (2013).

10.1126/science.1245475

CREDIT: H. MACDONALD/SCIENCE

Fueling Immunity: Insights into Metabolism and Lymphocyte Function

Erika L. Pearce,* Maya C. Poffenberger, Chih-Hao Chang, Russell G. Jones*

READ THE FULL ARTICLE ONLINE

<http://dx.doi.org/10.1126/science.1242454>



Cite this article as Pearce *et al.*, *Science* 342, 1242454 (2013). DOI: 10.1126/science.1242454

Background: Naïve lymphocytes circulate in the body in a resting state, but upon recognition of foreign antigen and receipt of proper costimulatory signals, these cells become activated, undergo a rapid burst in proliferation, and assume effector functions aimed at controlling or killing the invader. There is a growing appreciation that changes in peripheral T cell function are not only supported by but are dependent on metabolic reprogramming and that specific effector functions cannot proceed without adopting the correct metabolism. However, the reasons underlying why T cells adopt specific metabolic programs and the impact that these programs have on T cell function and, ultimately, immunological outcome remain unclear.

Advances: Research into the metabolism of tumor cells has provided valuable insight into the metabolic pathways important for cell proliferation and survival, as well as the influence of metabolites themselves on signal transduction and epigenetic programming. Many of these concepts have shaped how we view metabolism in T cells. However, it is important to note that, unlike tumors, T cells rapidly transition between resting catabolic states (naïve and memory T cells) to one of growth and proliferation (effector T cells) as part of a normal developmental program. In addition, as T cells differentiate during an immune response, they also move from what are presumably nutrient-replete lymphoid organs to sites of cancer or infection, where oxygen, nutrients, and growth factors may become limiting. Thus, T cells must metabolically adapt to these changing conditions in order to perform their necessary functions. In this Review, we highlight emerging areas in the metabolism of these dynamic cells and discuss the potential impact of metabolic control on T cell fate, plasticity, and effector function.

Outlook: It is becoming increasingly clear that T cell function is intimately linked to metabolic programs, and as such there is a considerable and growing interest in developing techniques that target metabolism for immunotherapy. Studying metabolism has often been difficult for the non-expert, because many of the experimental approaches require specialized instrumentation that has not been widely available. Furthermore, acquiring sufficient cellular material for ex vivo analyses, coupled with the inherent difficulty of assessing cellular metabolism in vivo during an immune response, presents substantial challenges to scientists studying the metabolism of immune cells. Nevertheless, understanding how environmental cues and cellular metabolism influence the outcome of T cell-mediated immune responses will be critical for learning how to exploit metabolism to alter disease outcome. Overall, we are just beginning to understand the pathways that regulate metabolism in lymphocytes and how T cells adapt to changes in their microenvironment, particularly in vivo; this area of immunology is poised for substantial advances in the years to come.

ARTICLE OUTLINE

Differential Regulation of T Cell Metabolism

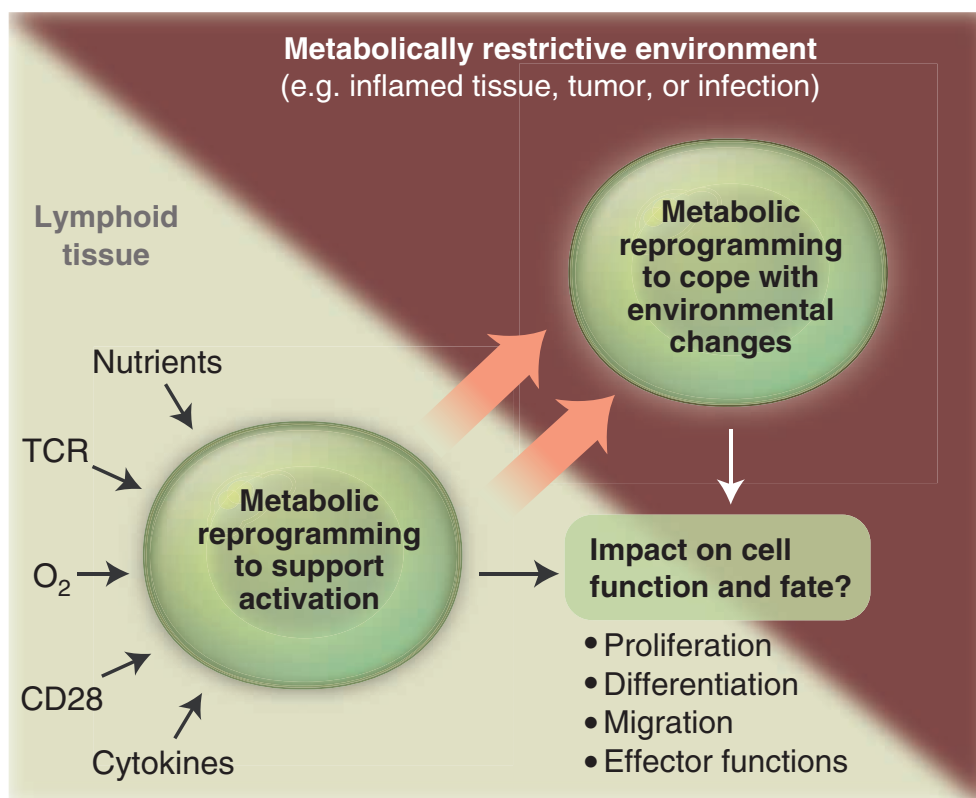
Metabolism of Proliferating Cells: Lessons from Tumor Metabolism

Metabolites As Signaling Molecules

Future Challenges and Other Considerations

Concluding Thoughts

T cell function and fate are dependent on metabolic reprogramming. As T cells differentiate during an immune response, they move from what are presumably nutrient-replete lymphoid organs to sites of cancer or infection, where oxygen, nutrients, growth factors, and other signals may become limiting. These metabolically restrictive environments force T cells to metabolically adapt in order to survive and perform their necessary functions.



The list of author affiliations is available in the full article online.

*Corresponding author. E-mail: erikapearce@path.wustl.edu (E.L.P.); russell.jones@mcgill.ca (R.G.J.)

Fueling Immunity: Insights into Metabolism and Lymphocyte Function

Erika L. Pearce,^{1*} Maya C. Poffenberger,^{2,3} Chih-Hao Chang,¹ Russell G. Jones^{2,3*}

Lymphocytes face major metabolic challenges upon activation. They must meet the bioenergetic and biosynthetic demands of increased cell proliferation and also adapt to changing environmental conditions, in which nutrients and oxygen may be limiting. An emerging theme in immunology is that metabolic reprogramming and lymphocyte activation are intricately linked. However, why T cells adopt specific metabolic programs and the impact that these programs have on T cell function and, ultimately, immunological outcome remain unclear. Research on tumor cell metabolism has provided valuable insight into metabolic pathways important for cell proliferation and the influence of metabolites themselves on signal transduction and epigenetic programming. In this Review, we highlight emerging concepts regarding metabolic reprogramming in proliferating cells and discuss their potential impact on T cell fate and function.

The immune system is comprised of a series of specialized cells conditioned to respond rapidly to “danger” signals such as foreign pathogens or inflammatory stimuli. T lymphocytes, or T cells, are sentinels of the adaptive immune system that respond to antigen-specific signals by blasting, proliferating, and differentiating into effector subsets tailored to identify and eliminate threats to the host. Integrated into this program of activation is the regulation of cellular metabolism. Upon activation, T cells dramatically alter their metabolic activity to meet the increased metabolic demands of cell growth, proliferation, and effector function. Metabolism fundamentally underpins T cell function; thus, there is great interest in understanding how metabolic pathways influence immune responses and ultimately affect disease progression. It should be noted that “metabolism” refers to a complex network of biochemical reactions involved in energy production and macromolecular biosynthesis, and comprehensive coverage of such a broad topic is difficult. Several recent reviews have highlighted the molecular mechanisms that govern metabolic reprogramming in the immune system (1–3). This Review will focus on emerging areas in intermediary metabolism in lymphocytes and will discuss their potential impact on T cell fate, plasticity, and effector function.

Differential Regulation of T Cell Metabolism

Lymphocyte Metabolism Is Dynamically Regulated

Maintenance of cellular bioenergetics is an essential function of all living cells, and lymphocytes are no exception. In T lymphocytes, glucose is

a critical substrate for adenosine triphosphate (ATP) production (4). During glycolysis, glucose is broken down into two molecules of pyruvate. This process, which does not require oxygen, yields two reduced nicotinamide adenine dinucleotide (NADH) molecules and two net ATP molecules per molecule of glucose. Pyruvate has two alternate fates. Most terminally differentiated,

nonproliferating cells can fully oxidize pyruvate in the tricarboxylic acid (TCA) cycle. This process generates NADH and reduced flavin adenine dinucleotide (FADH₂), which the cell can use to fuel OXPHOS, an oxygen-dependent process that produces up to 36 molecules of ATP per glucose molecule. Alternatively, pyruvate can be transformed (or fermented) into lactate, regenerating NAD⁺ for subsequent use in glycolysis (5). From a bioenergetic perspective, engaging OXPHOS maximizes the amount of ATP that can be derived from glucose.

Bioenergetic profiling of T cells has revealed that T cell metabolism changes dynamically with activation state (Fig. 1). Upon antigen encounter, T cells become activated, undergo extensive proliferation, and differentiate into effector T cells (T_{EFF}); upon pathogen clearance, most T_{EFF} cells die, leaving behind a small population of long-lived antigen-specific memory T cells (T_M). Consistent with the metabolism of other nonproliferating cells, resting naïve T cells (T cells that have not yet encountered antigen) maintain low rates of glycolysis and predominantly oxidize glucose-derived pyruvate via OXPHOS or engage fatty acid oxidation (FAO) to make ATP. Upon activation, T cells switch to a program of anabolic growth and biomass accumulation to generate daughter cells,

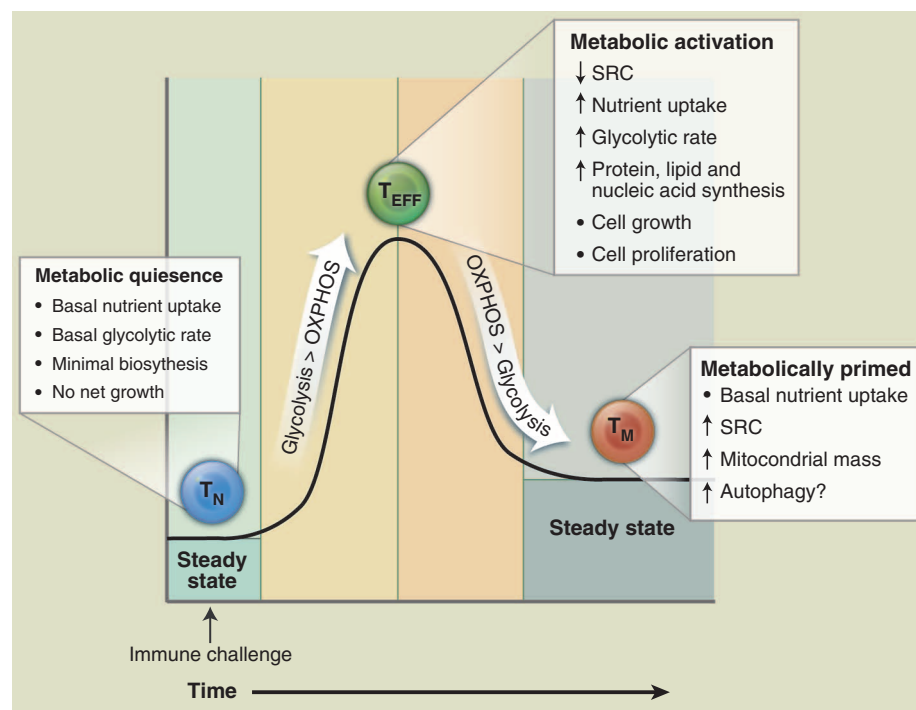


Fig. 1. T cell metabolism changes over the course of an immune response. T cells display distinct metabolic profiles depending on their state of activation. Naïve T cells (T_N, blue) are metabolically quiescent; they adopt a basal level of nutrient uptake and use OXPHOS as their primary pathway of ATP production. Upon immune challenge, T_{EFF} (green) cells shift to a state of metabolic activation characterized by increased nutrient uptake, elevated glycolytic and glutaminolytic metabolism, biomass accumulation, and reduced mitochondrial SRC. T_{EFF} cells preferentially use glycolysis over OXPHOS for ATP production. Transition to the T_M (orange) stage is characterized by a quiescent metabolism, with increased reliance on FAO to fuel OXPHOS. Mitochondrial mass and SRC are elevated in T_M cells, suggesting that these cells are metabolically primed to respond upon reinfection.

¹Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110, USA. ²Goodman Cancer Research Centre, McGill University, Montreal, QC H3G 1Y6, Canada. ³Department of Physiology, McGill University, Montreal, QC H3G 1Y6, Canada.

*Corresponding author. E-mail: erikapearce@path.wustl.edu (E.L.P.); russell.jones@mcgill.ca (R.G.J.)

which by definition dictates increased demand for ATP and metabolic resources. In this state, T cells are considered to be metabolically activated (Fig. 1). T cell receptor (TCR) signaling directs the metabolic reprogramming of naïve T cells. TCR ligation promotes the coordinated up-regulation of glucose and amino acid transporters (6–8), facilitating nutrient uptake and T cell blastogenesis. TCR-mediated up-regulation of the transcription factors c-Myc (9) and estrogen-related receptor α (ERR α) (10) enhances the expression of genes involved in intermediary metabolism. In addition, catabolic pathways of ATP generation such as fatty acid β -oxidation are actively suppressed (9). The predominant metabolic phenotype of activated T cells is a shift to aerobic glycolysis [reviewed in (11)]. Both CD4⁺ and CD8⁺ T_{EFF} cells engage aerobic glycolysis, which is marked by the conversion of glucose-derived pyruvate to lactate despite the availability of oxygen for complete glucose oxidation. This process, also known as the Warburg effect from earlier work in cancer biology, is a common trait of actively proliferating cells (5). It is important to note that OXPHOS is still engaged in T_{EFF} cells (9); however, the production of lactate from pyruvate by aerobic glycolysis is the dominant pathway of glucose metabolism in T_{EFF} cells. Regulating energy metabolism may provide a way for T cells to reversibly switch between quiescent and highly proliferative states (12).

As a quiescent T cell population, T_M cells adopt a metabolic profile similar to that of naïve T cells—a catabolic metabolism characterized by increased reliance on OXPHOS and lower rates of nutrient uptake and biosynthesis relative to T_{EFF} cells (Fig. 1). However, T_M cells also display a characteristic increase in mitochondrial mass, which translates into greater mitochondrial spare respiratory capacity (SRC) relative to naïve or T_{EFF} populations (13). SRC can be viewed as the maximal respiratory capacity available to a cell, much like the maximum speed that can be achieved by a car engine. Under increased workload, stress, or nutrient limitation, cells engage this reserve capacity to generate more energy and promote cell viability (14, 15). We have recently shown that increased mitochondrial mass and SRC of T_M cells allows for rapid mitochondrial ATP production upon TCR engagement, conferring a bioenergetic advantage to T_M cells upon secondary exposure to antigen (16). From this vantage, T_M cells may be viewed as being metabolically primed, with mitochondrial metabolism fueling the rapid recall response to reinfection. The memory T cell–promoting cytokine interleukin (IL)–15 plays a key role in this catabolic switch by promoting mitochondrial biogenesis (13).

The mechanisms governing the transition of T cells from effector to memory states are still poorly understood, but recent work hints that changes in metabolism may influence this process. We previously demonstrated that mitochondrial FAO stimulated downstream of TNF (tumor necrosis factor) receptor–associated factor 6 (TRAF6)

is required for memory CD8⁺ T cell development (17). Oxidation of free fatty acids (FFAs) generates acetyl–coenzyme A (CoA), which can be metabolized further in the TCA cycle, as well as FADH₂ and NADH, which can be used directly by the electron transport chain (ETC) to make ATP. FFAs are energy-dense molecules, and FAO may be a preferred fuel source for T_M cells as they rely on OXPHOS-dependent metabolic program. Administration of metformin, a metabolic stressor that activates the energy sensor adenosine monophosphate–activated protein kinase (AMPK), enhances the generation of CD8⁺ T cell memory (17). One consequence of AMPK activation is the suppression of mammalian target of rapamycin complex 1 (mTORC1) activity in response to energetic stress (18). Consistent with this, the drug rapamycin, which also inhibits mTORC1, enhances the generation of CD8⁺ T_M cells (17, 19, 20). These observations suggest that manipulating the metabolism of antigen-specific cells during contraction can influence the development of T_M cells. Given these observations, T_M formation may be influenced by a number of enzymes and transporters involved in fatty acid synthesis, desaturation, and oxidation, as well as the availability of FFAs to memory precursor cells. Some important players to consider in this regard include acetyl-CoA carboxylase (ACC2) (21), the mitochondrial lipid transporter CPT1A (13, 22), and metabolites such as acetyl-CoA and malonyl-CoA (23). AMPK activation and mTOR inhibition are also both potent activators of autophagy, a catabolic process induced during starvation that promotes the degradation and recycling of cellular components [reviewed in detail in (24)]. Proper induction of autophagy has been shown to be important for the maintenance of cellular bioenergetics and sustained T cell viability after activation (25, 26). It will be interesting to determine whether autophagy, by coupling catabolic fuel supply to mitochondrial metabolism, is important for T_M formation after infection.

Mitochondrial OXPHOS and T Cell Activation

Although much focus has been placed on the shift toward glycolysis that accompanies T cell activation, evidence suggests that mitochondrial OXPHOS is also important for T cell activation. Oligomycin, a specific inhibitor of mitochondrial ATP synthase, can block the expression of early activation markers after TCR ligation and blunts subsequent T cell proliferation (27), suggesting that the naïve-to-effector transition requires either de novo production of ATP by mitochondria or specific signals generated during mitochondrial ATP production. Mitochondrial-derived reactive oxygen species (ROS) may function as such a “bioenergetic” second messenger. There has long been evidence that ROS can play critical roles in shaping T cell responses (28–30). However, recent work suggests that mitochondrial ROS produced during OXPHOS is essential for T cell activation. T cells deficient for ubiquinol-cytochrome c reductase (Uqcrcf1), a component of complex III of

the ETC, display impaired TCR-dependent ROS production and defects in antigen-specific proliferation (31). Intracellular calcium (Ca²⁺) flux, an early event in TCR signal transduction, may provide the functional link between TCR ligation, mitochondrial OXPHOS, and cell proliferation. Uptake of Ca²⁺ by mitochondria stimulates Ca²⁺-dependent dehydrogenases of the TCA cycle, driving mitochondrial NADH production and ATP production by OXPHOS during early T cell activation (32). T cells lacking the apoptosis regulators Bax and Bak, which display defects in intracellular Ca²⁺ homeostasis, exhibit reduced Ca²⁺-dependent mitochondrial ROS production and T cell proliferation after TCR stimulation (33). Restoring Ca²⁺ signals in Bax/Bak-null T cells restores mitochondrial ROS production and T cell proliferation (33). Thus, although toxic in many biological settings, mitochondrial-dependent ROS may prime T cells and license full T cell activation.

Metabolic Signatures Vary with Differentiation State

Although the paradigm of T cell metabolism as summarized in Fig. 1 holds true with respect to activated versus quiescent states, the metabolic signature of T cells can also vary depending on differentiation state. This was first demonstrated by Michalek *et al.* (34), who determined that proinflammatory CD4⁺ T helper (T_H) cells (T_H1, T_H2, and T_H17 lineages) displayed a strong bias toward glycolysis over mitochondrial metabolism, whereas induced CD4⁺ T regulatory (T_{reg}) lineage cells displayed a mixed metabolism involving glycolysis, lipid oxidation, and OXPHOS. In particular, T_H17 cells display increased reliance on glycolysis for their development and maintenance. T_H17 cell development is promoted by hypoxia inducible factor–1 α (HIF-1 α) (35, 36), an oxygen-sensitive transcription factor that regulates glycolytic gene expression in T_H17 cells. Blocking glycolysis during T_H17 cell differentiation reduced the development of T_H17 cells and favored the formation of T_{reg}s (35). Added to this are recent results indicating that extracellular salt (NaCl) (37, 38) and short-chain fatty acids (39) can influence T_H17 and T_{reg} homeostasis, respectively. This raises the intriguing possibility that the metabolic microenvironment (i.e., nutrient and oxygen availability) can influence T cell polarization (to be discussed later). Determining whether the metabolic signature of differentiated T cells is simply a consequence of lineage-specific cytokine signaling or is instructive for T cell function (i.e., essential for regulating T cell plasticity and/or effector function) remains a question for the field. Examining the influence of key metabolic regulators such as HIF-1 α , mTOR, and AMPK on T cell differentiation and plasticity will help in resolving these issues.

Metabolism of Proliferating Cells: Lessons from Tumor Metabolism

Research in cancer metabolism over the past 10 years has increased our understanding of the

metabolic requirements of proliferating cells, as well as the metabolic alterations that promote tumor growth. One of the great innovations we have learned from the cancer metabolism field is that signaling pathways, or more specifically the oncogenes and tumor suppressors that comprise and regulate signal transduction pathways, can influence cellular metabolism as part of their program of action. The previous paradigm of metabolic regulation argued that metabolic pathways were exclusively controlled through allosteric regulation of metabolic enzymes, either by ATP levels or metabolites themselves (the reactive model) (40). One example of this is the glycolytic enzyme phosphofructokinase (PFK), which is inhibited allosterically by ATP and citrate (indicating a high energy state in the cell) and stimulated by AMP (indicative of low energy). Although allosteric regulation is important for regulating local flux through metabolic pathways, we now understand that activation of signal transduction pathways (such as phosphatidylinositol 3-kinase or Akt) by growth factor receptors stimulates global changes in metabolic flux independent of ATP levels. This allows a cell that receives a proliferative signal, such as a T cell activated through its TCR, to drive cellular metabolism above the capacity normally maintained in the quiescent state. Overall, metabolism in T cells is likely regulated at several levels: (i) TCR-mediated changes in the expression of metabolic genes facilitate the reprogramming required to match metabolic pathways to biological need; (ii) signal transduction downstream of cell surface receptors (i.e., costimulatory molecules and cytokine receptors) serve to fine-tune flux through these metabolic pathways; and (iii) feedback inhibition and other forms of allosteric regulation can regulate metabolic flux through local nodes in the network. By directly influencing metabolic reprogramming, oncogenes and tumor suppressors gain control over the metabolic currency of the cell, namely, energetic intermediates (ATP, NAD^+/NADH , $\text{FAD}^+/\text{FADH}_2$, and $\text{NADP}^+/\text{NADPH}$) and metabolites involved in bioenergetic and biosynthetic reactions that influence cell growth and survival.

The implication of these findings for immunologists is that metabolic pathways are indirectly connected to cell surface receptors of the immune system via signal transduction pathways. TCR/CD28 stimulation of T cells (6), the stimulation of surface immunoglobulin on B cells (41), and TLR stimulation of macrophages and dendritic cells (DCs) (42, 43) all promote changes in aerobic glycolysis characteristic of the Warburg effect. These results likely just scratch the surface of the complex metabolic networks at work in proliferating cells. The challenge going forward will be to identify key pathways of metabolic flux integral for lymphocyte function. In this regard, research into tumor cell metabolism has provided valuable insight into the metabolic pathways important for cell proliferation. Many of the metabolic pathways abnormally activated in cancer,

such as aerobic glycolysis, have been shown to play similar roles in normal lymphocyte physiology. Here, we highlight recent advances in intermediate metabolism observed in cancer that are likely to be relevant to T cell biology.

The Warburg Effect: More than ATP Synthesis

Rapid glucose processing promoted by the Warburg effect allows proliferating T cells to generate ATP quickly; glycolysis also generates metabolic intermediates important for cell growth and proliferation (Fig. 2). Metabolism of glucose through the oxidative or nonoxidative arms of the pentose phosphate pathway (PPP) generates ribose-5-phosphate (Rib-5P), a key intermediate in nucleotide biosynthesis. The oxidative arm of the PPP also produces NADPH, the key metabolic currency for nucleotide and fatty acid biosynthesis. T cell activation promotes a rapid increase in glucose flux through the oxidative PPP (9). Dihydroxyacetone-phosphate (DHAP) is used to generate the glycerol backbone for glycerophospholipids, and 3-phosphoglycerate (3PG) is a key intermediate in both amino acid and nucleotide biosynthesis (discussed below). Pyruvate that is not converted to lactate can enter into the mitochondria and be converted into acetyl-CoA by the pyruvate dehydrogenase (PDH) complex. In proliferating cells, mitochondria adopt an additional role as a biosynthetic hub, converting pyruvate and other metabolites into metabolic intermediates involved in protein and fatty acid biosynthesis (44).

One of the key metabolic intermediates for biosynthesis is acetyl-CoA. Acetyl-CoA has a central role in membrane biogenesis because it provides two-carbon units for fatty acid and isoprenoid biosynthesis, as well as in other diverse processes such as protein prenylation and *N*-glycosylation (45). The flow of glucose to the cytosolic acetyl-CoA pool is regulated by using TCA cycle intermediates and a truncated TCA cycle (46). In this model, mitochondrial citrate is formed from condensation of oxaloacetate and acetyl-CoA, after which citrate is exported from the mitochondrion to the cytosol and converted back to acetyl-CoA by ATP citrate lyase (ACL) (47). Despite the availability of extracellular lipids for membrane biosynthesis, FFAs are generated *de novo* from glucose in proliferating cells using this pathway (46). CD8⁺ T cells unable to engage acetyl-CoA-dependent lipid biosynthetic pathways display defects in antigen-driven blastogenesis and clonal expansion in response to pathogens (48). Acetyl-CoA can also influence metabolic flux through acetylation of metabolic enzymes (49, 50), reinforcing metabolic pathways such as glycolysis when carbon availability is high. Glucose availability and acetyl-CoA production can also influence epigenetics by regulating the cytosolic acetyl-CoA pools available for histone acetylation reactions (51), raising the prospect that glucose-dependent metabolic flux may help drive or reinforce T cell differentiation programs.

One consequence of using glucose-derived mitochondrial citrate for lipid biosynthesis is the potential depletion of TCA cycle intermediates. Oxaloacetate (OAA) is a rate-limiting substrate for acetyl-CoA entry into the TCA cycle. OAA generated from ACL-mediated cleavage of cytosolic citrate can potentially cycle back into the mitochondria to maintain the TCA cycle. Inefficient cycling of this pathway would lead to cumulative depletion of mitochondrial OAA, leading to collapse of the TCA cycle and disruption of mitochondrial function. One way tumor cells counter this is by engaging glutaminolysis, a metabolic shunt that converts glutamine into α -ketoglutarate (α -KG, also known as 2-oxoglutarate) for use in the TCA cycle (Fig. 2). Glutamine has long been known as a key metabolite for supporting T cell function (52). Recent evidence suggests that glutamine metabolism is as dynamically regulated in T cells as glucose metabolism. Glutamine transporters (SNAT1 and SNAT2) as well as key glutaminolysis enzymes (GLS, GPT, GOT, and GLUD) are up-regulated early after T cell activation similar to glycolysis genes (7, 9); several groups have correlated these changes in gene expression to enhanced glutaminolytic flux in lymphocytes (9, 53). Recently, it was found that engagement of the TCR leads to the expression of Slc7a5, an amino acid transporter that mediates the import of large neutral amino acids, such as leucine (8). Amino acid influx via this transporter is required for the activation of mTOR and expression of c-Myc and as such coordinates activation-induced metabolic reprogramming and differentiation of T cells. Thus, amino acids such as glutamine and leucine appear to play additional roles in T cell function beyond protein biosynthesis and may directly influence T cell activation by regulating metabolic reprogramming.

Control of Glycolytic Flux by Pyruvate Kinase M2 (PKM2)

Pyruvate kinase (PK) is a key enzyme of the glycolytic pathway. It catalyzes the terminal reaction of glycolysis by promoting the conversion of phosphoenolpyruvate (PEP) to pyruvate (Fig. 2), and is one of two ATP-generating steps of glycolysis (the second is mediated by phosphoglycerate kinase). The muscle version of PK exists as one of two isoforms, M1 or M2, generated from differential splicing of the *PKM* primary mRNA transcript, with the PKM2 splice variant expressed in embryonic tissues, proliferating cells, and tumor cells (54). Naïve T cells express both M1 and M2 isoforms at rest; mitogen-dependent activation promotes the rapid accumulation of the M2 isoform, which becomes the dominant isoform expressed in T_{EFF} cells (55). PKM2 exists as either an inactive dimer or an active tetramer, and oscillation between these two states influences the ability of cells to maintain anabolic metabolism (56).

Multiple lines of evidence point to a role for PKM2 in coordinating glycolytic flux and cell

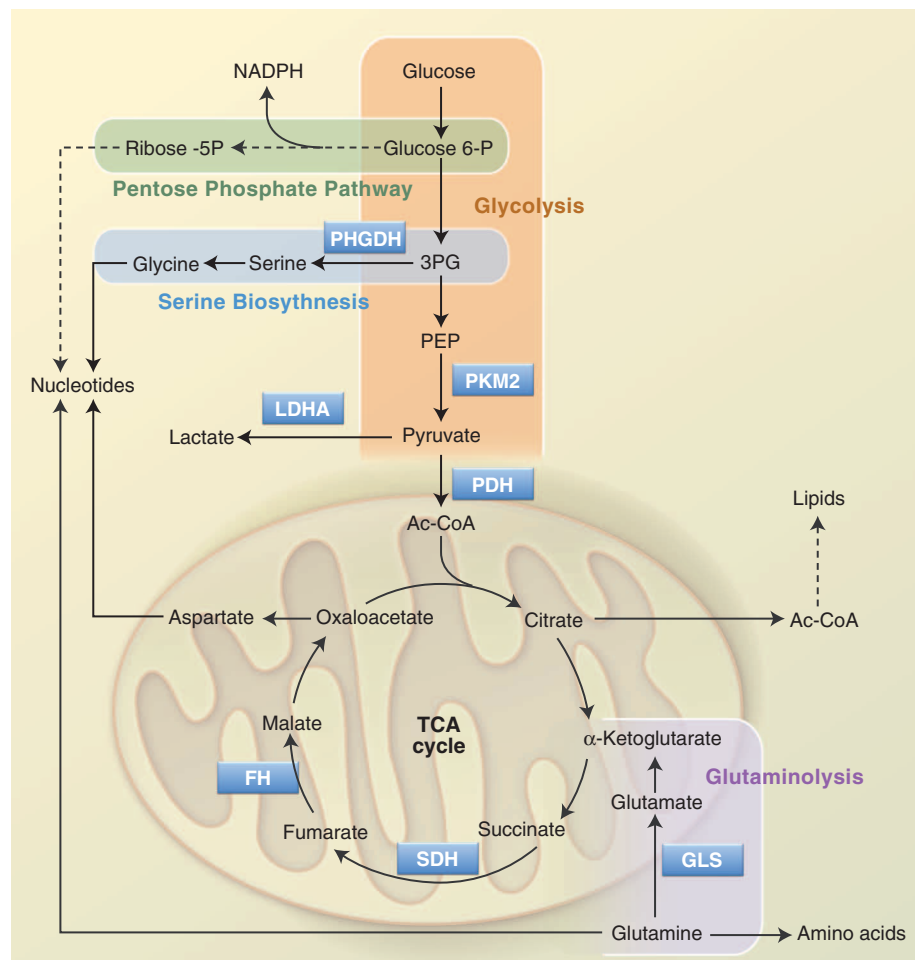


Fig. 2. Metabolic pathways that support cell growth and proliferation. Glycolysis and the TCA cycle are two separate yet connected biochemical pathways that function to generate ATP as well as metabolic precursors for biosynthesis. Glucose is broken down to pyruvate by glycolysis (orange); pyruvate can be further oxidized by the TCA cycle in the mitochondrion. Glycolytic intermediates can be used to generate other metabolites required for growth and proliferation. Glucose 6-phosphate and 3PG produced from glycolysis are metabolized in the PPP (green) and the SBP (blue), respectively, providing important precursors for nucleotide biosynthesis. Similarly, acetyl-CoA, generated from glucose-derived citrate in the TCA cycle, can be used for lipid biosynthesis. OAA, produced as part of the TCA cycle, can be used to generate aspartate, another precursor for nucleotide synthesis. An alternate source of carbon for the TCA cycle occurs via glutaminolysis (purple); in this pathway, glutamine is converted to glutamate and then to α -KG, which joins the TCA cycle. Glutamine is also a precursor for amino acid and nucleotide biosynthesis. Key enzymes in these pathways are PHGDH; PKM2; LDHA, lactate dehydrogenase; PDH; GLS, glutaminase; SDH, succinate dehydrogenase; and FH.

proliferation. Tumor cells engineered to exclusively express the M2 isoform display increased lactate production characteristic of the Warburg effect and gain a growth advantage *in vivo* over M1-expressing tumors (57). Interestingly, the M2 isoform is actually less efficient at converting PEP to pyruvate than the M1 isoform. Moreover, growth-factor-stimulated tyrosine phosphorylation of PKM2 further decreases its activity (58, 59). This prompts the question: Why would proliferating cells, including T cells, promote the expression of a pyruvate kinase isoform that is less efficient at generating ATP? The answer to this question may lie with the second function of Warburg metabolism, namely, supporting anabolic growth. Buildup of PEP because of reduced PKM2 ac-

tivity promotes the accumulation of glycolytic intermediates, which can then be shunted into upstream biosynthetic pathways to support amino acid, triglyceride, and nucleotide biosynthesis. Activating PKM2 by using small-molecule agonists increases PEP-to-pyruvate conversion, reducing the flux of glycolytic intermediates toward anabolic pathways and slowing tumor cell growth (56, 60). Thus, the ability of PKM2 to support cell proliferation may have less to do with ATP production and more with supporting biosynthetic pathways required for tumor cell growth.

PKM2 may also exert some of its effects on cell proliferation through nonmetabolic functions, including transcriptional and epigenetic regulation (61, 62). Of particular interest to immu-

nologists is that PKM2 can phosphorylate signal transducer and activator of transcription 3 (STAT3) at Tyr⁷⁰⁵, promoting increased STAT3-dependent transcription (63). The phosphorylation of STAT3 by PKM2 demonstrates that PKM2 possesses both protein and pyruvate kinase activities, the former using PEP as a phosphate donor rather than ATP. The protein kinase activity of PKM2 (and any subsequent effects on STAT3 activity) would be predicted to be sensitive to metabolic flux, favored under high-glycolysis PEP conditions and antagonized by low-energy high-ADP concentrations. The development of mouse models to study the impact of PKM2 activity *in vivo* will be important for elucidating the role(s) of PKM2 in immune function.

The Serine Biosynthesis Pathway

Another glycolytic intermediate that can double as an anabolic precursor is 3PG. 3PG is the starting point for the glucose-dependent biosynthesis of serine and glycine via the serine biosynthesis pathway (SBP) (Fig. 3A). Key enzymes of the SBP are phosphoglycerate dehydrogenase (PHGDH), the rate-limiting step for serine biosynthesis, and serine hydroxymethyltransferase (SHMT), which uses serine as a methyl donor to convert tetrahydrofolate (THF) to methylene-THF, generating glycine in the process. Methylene-THF is a key intermediate in folate-mediated one-carbon metabolism that fuels nucleotide biosynthesis and methylation reactions. Serine is also an allosteric activator of PKM2 (64) and thus provides feedback to the glycolytic pathway to regulate 3PG levels and serine biosynthesis.

In mammals, serine and glycine are non-essential amino acids and are widely abundant in serum (and tissue culture medium). However, glucose-dependent serine biosynthesis is actively engaged in some tumors regardless of serine abundance. Amplification of *PHGDH* has been observed in breast cancer and melanoma (65), and increased *PHGDH* expression can promote both enhanced serine biosynthesis and the proliferation of cancer cells (65, 66). Enhanced flux through the SBP may confer a growth advantage to tumor cells beyond providing increased serine and glycine for biosynthetic reactions. First, *PHGDH* produces NADH, which can be used to maintain cytosolic redox balance or fuel mitochondrial OXPHOS to make ATP. The second step of the pathway, the conversion of 3-phosphohydroxypyruvate to 3-phosphoserine by phosphoserine aminotransferase (PSAT), requires glutamate and produces α -KG (Fig. 3A) (66). Thus, the SBP may promote an alternate pathway of α -KG production for mitochondrial metabolism or promote the activity of α -KG-dependent enzymes (to be discussed later). Last, serine and glycine are both intermediates in the production of reduced glutathione (GSH), a key cellular antioxidant. Recent evidence indicates that cancer cells actively produce GSH from glucose via the SBP as a buffer against oxidative damage (67). The expression of SBP enzymes is

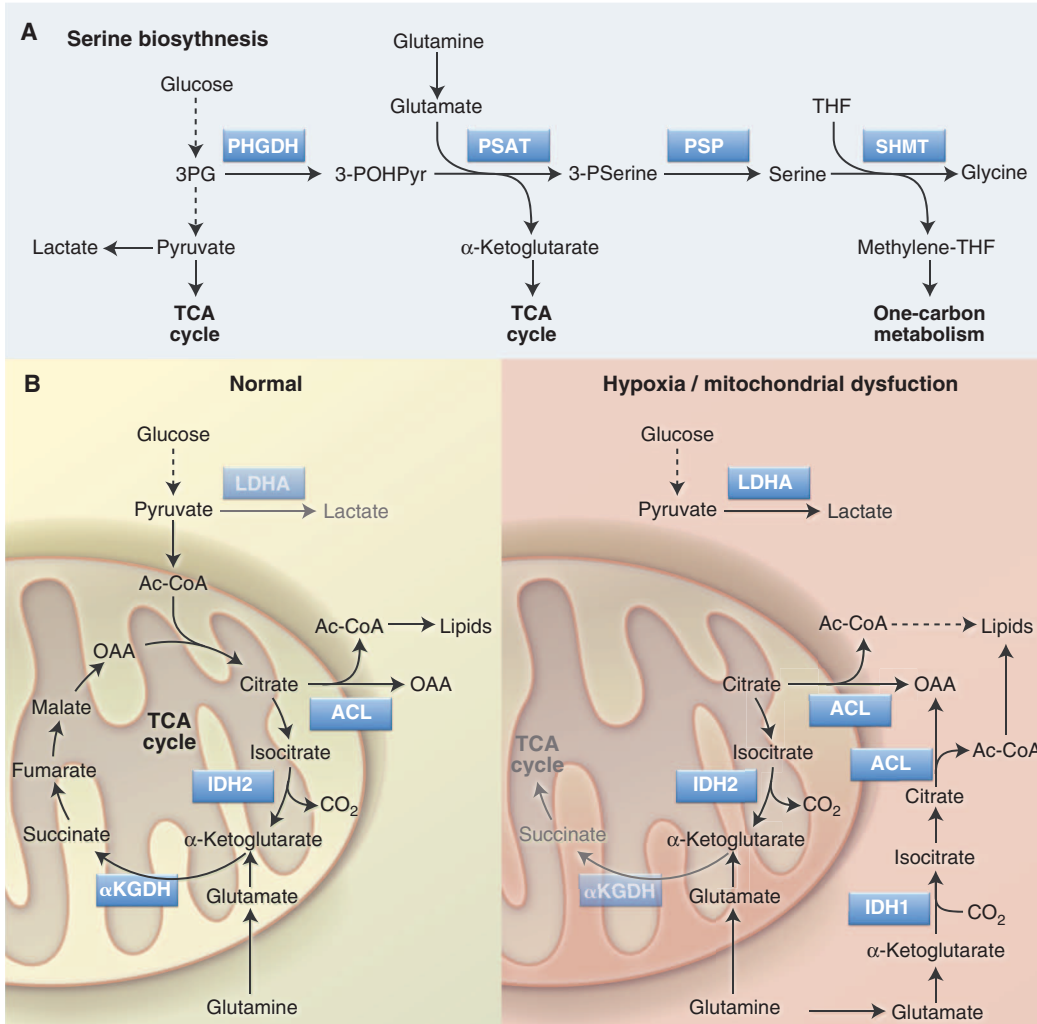


Fig. 3. Serine biosynthesis and reductive carboxylation are anabolic pathways that support cell proliferation. (A) The SBP converts glucose-derived 3PG into serine and glycine, which are precursors for lipid and nucleotide biosynthesis. Serine is also involved in folate-mediated one carbon metabolism by acting as a methyl group donor for THF to methylene-THF conversion. Key enzymes in this pathway are PHGDH, PSAT, and SHMT. (B) Reductive carboxylation is an alternate pathway of glutamine metabolism in which glutamine-derived α -KG is converted to citrate through reverse TCA cycle flux. Under conditions of hypoxia or mitochondrial dysfunction (right), isocitrate dehydrogenase (IDH1 in cytosol, IDH2 in mitochondria) uses CO_2 and NADPH to convert α -KG into isocitrate. Citrate produced downstream of this reaction is converted into cytosolic acetyl-CoA without passing through the conventional clockwise steps of the TCA cycle. Acetyl-CoA generated by this pathway can function as a precursor for fatty acid synthesis. α -KGDH, α -KG dehydrogenase.

up-regulated in Myc-driven lymphomas (68); thus, we hypothesize that T_{EFF} cells may actively engage the SBP upon activation, even in the presence of exogenous serine. Future investigation will be needed to determine how the SBP influences T cell biology.

Reductive Carboxylation of α -KG

As mentioned previously, it is now appreciated that in proliferating cells the TCA cycle functions as a source of biosynthetic precursors in addition to its role in ATP production (46). Carbon enters the TCA cycle primarily at one of two entry points: (i) the condensation of glucose-derived acetyl-CoA with OAA to generate citrate and (ii) conversion of glutamine to α -KG via glutaminolysis (Fig. 3B). Carbon-tracing experiments using pro-

liferating glioblastoma cells have established that both glucose and glutamine contribute to mitochondrial citrate pools and subsequent lipid synthesis (69). The oxidative decarboxylation of isocitrate to α -KG by isocitrate dehydrogenase (IDH) is highly favored thermodynamically, such that this reaction is believed to be irreversible and the reason for the “clockwise” flow of metabolic intermediates through the TCA cycle.

Groundbreaking new work suggests that metabolite flow through biochemical pathways does not always conform to conventional dogma. Although oxidation of glutamine-derived α -KG in the TCA cycle serves as a minor source of lipogenic acetyl-CoA under normal growth conditions, α -KG can be converted to citrate through reductive carboxylation under conditions of stress

such as hypoxia or mitochondrial dysfunction (70–72). In this reaction, glutamine-derived α -KG is carboxylated, rather than decarboxylated, by IDH1 (in the cytosol) or IDH2 (in mitochondria) to generate citrate (Fig. 3B). This switch to reductive versus oxidative metabolism of α -KG is regulated in part by HIF-1 α (71, 72), although the exact mechanism by which HIF-1 α does so remains unclear. Engaging reductive carboxylation of α -KG in essence bypasses the conventional TCA cycle by using glutamine to generate the acetyl-CoA required for fatty acid synthesis (Fig. 3B). Under such metabolic reprogramming, cancer cells continue to use glycolysis for ATP production but switch from glucose to glutamine as the major lipogenic precursor.

One implication of this work is that tumor cells display a high degree of metabolic plasticity and can adapt their metabolism to support proliferation and viability under fluctuating environmental conditions. Whether T cells display similar metabolic flexibility in response to environmental cues requires further investigation. The work from Metallo and colleagues (71) suggests that T cells can use reductive glutamine metabolism for fatty acid biosynthesis under hypoxic conditions. Physiologic oxygen tension varies significantly between tissues, and hypoxic regions can be detected in the bone marrow, thymus, and spleen (73). Moreover, T cells are likely to experience hypoxia at sites of tissue inflammation (74). Stabilization of HIF-1 α and metabolic reprogramming to favor reductive carboxylation may help T cells maintain proliferation and/or effector function at hypoxic inflammatory sites. HIF-1 α

protein expression has also been observed early after T cell activation (75), so it is unclear whether reductive carboxylation may also be engaged as part of the normal program of T cell expansion. As mentioned, HIF-1 α has been implicated in the differentiation of both proinflammatory T_H17 cells (35, 36) and $CD4^+ \text{FoxP3}^+ T_{\text{reg}}$ cells (76). It is tempting to speculate that this alternate pathway of glutamine metabolism may influence the expansion or phenotypic stability of these T cell subsets.

Metabolites As Signaling Molecules

Cancer genome sequencing efforts yielded an unexpected discovery in 2008 with the identification of somatic mutations in a metabolic enzyme, the TCA cycle enzyme IDH1, in glioblastoma

multiforme (GBM) (77). Mutations in IDH1 presented at significant frequency (~12% of GBM patients) with high frequency of missense mutations targeting an arginine residue in the active enzyme site (Arg¹³²). This mutation alters the enzymatic ability of IDH1, allowing it to convert α -KG to 2-hydroxyglutarate (2-HG) rather than promote normal isocitrate-to- α -KG interconversion (78). In the cancer setting, 2-HG has been shown to influence a number of biological processes, including cell differentiation, DNA methylation, and histone methylation (79–82), leading to its classification as an oncometabolite. These results have shifted thinking in cancer biology to consider that specific metabolites may also act as signaling molecules to influence cell physiology. Given certain similarities between metabolic programming in tumor cells and proliferating T cells, it stands to reason that metabolites will participate in T cell signaling as well. Some examples of how metabolites shape T cell function and fate through metabolic pathways are discussed in detail here.

Regulation of LKB1-AMPK Signaling by Adenylates

ATP is the primary carrier of chemical energy in the cell and essential for life. Thus, adenylates [ATP, adenosine diphosphate (ADP), and AMP] are important units of cellular metabolic currency. In mammalian cells, fluctuations in cellular energy are monitored by the heterotrimeric AMPK complex [reviewed in (83)]. ATP, ADP, and AMP compete for nucleotide-binding sites of the γ regulatory subunit of AMPK, with AMP (low energy) promoting and ATP (high energy) antagonizing AMPK activation. As such, AMPK functions as a sensor of the cellular adenylate energy charge (84, 85). Elevation of the cellular AMP:ATP ratio leads to increased phosphorylation of AMPK at Thr¹⁷² of its activation loop by

the kinase LKB1 (Fig. 4A). AMPK can also be activated by Ca²⁺ (via CamKK β) and the cytokine transforming growth factor- β (TGF- β) (via TAK1), although LKB1 appears to be the sole kinase that couples adenylate binding to AMPK activation.

Together LKB1 and AMPK function as part of an evolutionarily conserved energy-sensing pathway that maintains cellular energy balance by promoting catabolic pathways of ATP production and limiting processes that consume ATP. Protein synthesis is one of the most energy-consuming processes in the cell, accounting for ~20% of cellular ATP consumption (86). As mentioned, AMPK antagonizes mRNA translation through negative regulation of mTORC1 (Fig. 4A) (18). By regulating AMPK activity, adenylates directly influence pathways of energy usage in the cell. As ATP levels drop, AMP binds to AMPK, and AMPK is switched on to promote ATP production and block its consumption; once ATP homeostasis has been reestablished, increased binding of ATP to AMPK shuts off the kinase.

Recent work indicates that LKB1-AMPK signaling can influence T cell metabolism and function. Lymphocytes exclusively express the $\alpha 1$ catalytic subunit of AMPK (87), and TCR stimulation promotes LKB1-dependent AMPK activation in lymphocytes (87, 88). Glycolysis is enhanced in resting AMPK α -deficient T cells (88), consistent with observations that silencing AMPK in tumor cells promotes the Warburg effect (89). Loss of LKB1-AMPK signaling promotes increased mTORC1 activity in both naïve and T_{EFF} cells (88, 90), which in turn facilitates production of the T_{H1} cytokine interferon- γ (IFN- γ) by T_{EFF} cells (88). Thus, LKB1 and AMPK act in concert to limit the anabolic growth of T cells by suppressing glycolysis and mTOR-dependent biosynthesis. Perhaps not surprisingly, deletion of either LKB1 or AMPK $\alpha 1$ disrupts normal lym-

phocyte homeostasis, resulting in an accumulation of activated (CD44^{hi} CD62L^{lo}) CD8⁺ T cells in animals (88). These results suggest that cellular adenylate levels and AMPK may help regulate lymphocyte pools in the whole organism.

Why would a signaling pathway that normally monitors cellular energy levels regulate T cell function? Similar to tumor cells (91), AMPK may regulate a metabolic checkpoint in T cells, acting as a brake on lymphocyte expansion when energy conditions are poor. T_{EFF} cells with defective AMPK signaling would be freed from these metabolic checkpoints and continue to proliferate and produce cytokines as if cellular bioenergetics were suitable to support T cell function. Additionally, AMPK may regulate the metabolic plasticity of lymphocytes, coordinating metabolic changes in response to nutrient fluctuation and allowing T cells to survive changes in their environment. As evidence for this, the AMPK agonist metformin, which promotes FAO in activated T cells, enhances the production of CD8⁺ memory T cells *in vivo* (17). Furthermore, it was recently shown that AMPK-deficient T cells are defective in their ability to generate CD8⁺ memory T cells during infection (92). There is also a growing body of evidence implicating AMPK in the control of inflammation (3). Future work will focus on the role of AMPK in regulating the metabolic fitness of lymphocytes, dissecting LKB1- and AMPK-specific effects on immune function and investigating the role(s) of LKB1 and AMPK in regulating inflammation *in vivo*.

Regulation of α -Ketoglutarate-Dependent Enzymes by TCA Cycle Metabolites

Although TCA cycle metabolites play central roles in energy metabolism, many function as chemical intermediates in other biological reactions. For example, fumarate can be used to chemically modify cysteine residues of proteins, resulting

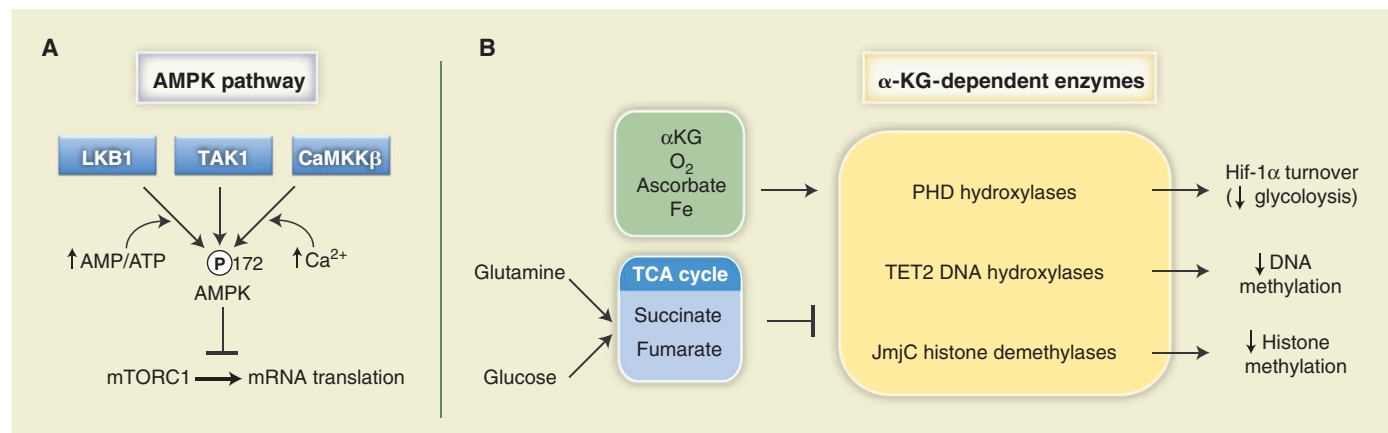


Fig. 4. Metabolites can influence signal transduction. (A) The AMPK pathway is influenced by adenylate concentration. AMPK α is activated by phosphorylation on Thr¹⁷² of its activation loop by the kinases LKB1, TAK1, or CaMKK β . LKB1 promotes enhanced AMPK phosphorylation under a high AMP:ATP ratio. One biological output of AMPK activity is the inhibition of mRNA translation under low-energy conditions through inhibition of mTORC1 activity. (B) α -KG-dependent enzymes (in yellow) are a class of enzymes regulated by TCA cycle

intermediates that require molecular oxygen (O₂) and α -KG for their enzymatic activity. Oxygen, α -KG, ascorbate, and iron (green) are positive regulators of these enzymes, whereas the TCA cycle intermediates fumarate and succinate (blue) antagonize their reactions. PHDs destabilize HIF-1 α protein, resulting in decreased expression of HIF-1 α targets and a reduction in glycolysis. TET2 hydroxylates 5-methylcytosine residues to promote DNA demethylation, whereas JmjC promotes demethylation of trimethylated histones in chromatin.

in the formation of S-(2-succinyl)cysteine or succinylation of these residues (93). Cancer cells harboring mutations in fumarate hydratase (FH), the TCA cycle enzyme that converts fumarate to malate, accumulate intracellular fumarate and display increased amounts of succinylated proteins (94). Increased protein succinylation has been associated with renal carcinoma and mechanistically can influence signal transduction pathways similar to protein phosphorylation or acetylation (95).

α -KG is another key metabolite involved in non-TCA cycle biochemical reactions. α -KG is essential for the activity of a class of enzymes known as α -KG-dependent dioxygenases (Fig. 4B). These enzymes use α -KG along with molecular O_2 , iron, and ascorbate to modify target proteins (96). The most extensively characterized enzymes of this family are the HIF prolyl hydroxylases (PHDs). PHDs use O_2 to hydroxylate HIF-1 α on two conserved proline residues, facilitating their recognition by the E3 ubiquitin ligase VHL and promoting HIF-1 α degradation by the proteasome. When O_2 availability is low, HIF-1 α protein is stabilized because of reduced PHD activity, resulting in increased HIF-1 α -dependent transcription (97). This regulatory circuit allows HIF-1 α to promote glycolytic ATP production when O_2 cannot support mitochondrial OXPHOS, an example of metabolic adaptation in response to environmental conditions. Other α -KG-dependent enzymes using this chemistry are the TET family of DNA hydroxylases, which hydroxylate 5-methylcytosine residues to promote DNA demethylation, and the Jumonji C (JmjC) class of histone demethylases (Fig. 4B). A detailed summary of these processes has recently been reviewed (98).

The activity of α -KG-dependent enzymes is directly affected by the abundance of TCA cycle intermediates. For example, 2-HG acts as a competitive inhibitor of α -KG, and its production by mutant IDH1 consumes α -KG (81, 99), leading to reduced TET2 and JmjC activity in tumor cells (79, 80). High levels of succinate and fumarate can inhibit PHD2 activity through product-mediated inhibition of PHD function, leading to HIF-1 α protein stabilization under normoxic (20% O_2) conditions (100, 101). Additionally, total abundance of α -KG is low in most cell types. The implication of these findings is that metabolic flux through the TCA cycle can affect gene transcription and/or epigenetic programs. It was recently shown that succinate plays a central role in production of the cytokine IL-1 β by lipopolysaccharide (LPS)-stimulated macrophages (102). LPS induces the de novo production of succinate from glutamine, leading to PHD inhibition, stabilization of HIF-1 α , and increased HIF-1 α -dependent IL-1 β production. Mutant IDH2 [Arg¹⁴⁰→Gln¹⁴⁰ (R140Q)], which promotes α -KG depletion/2-HG production, promotes DNA hypermethylation in hematopoietic cells and can inhibit myeloid differentiation when overexpressed in bone marrow stem cells (79). Differentiation of T cells to specific T_H lineages is driven by spe-

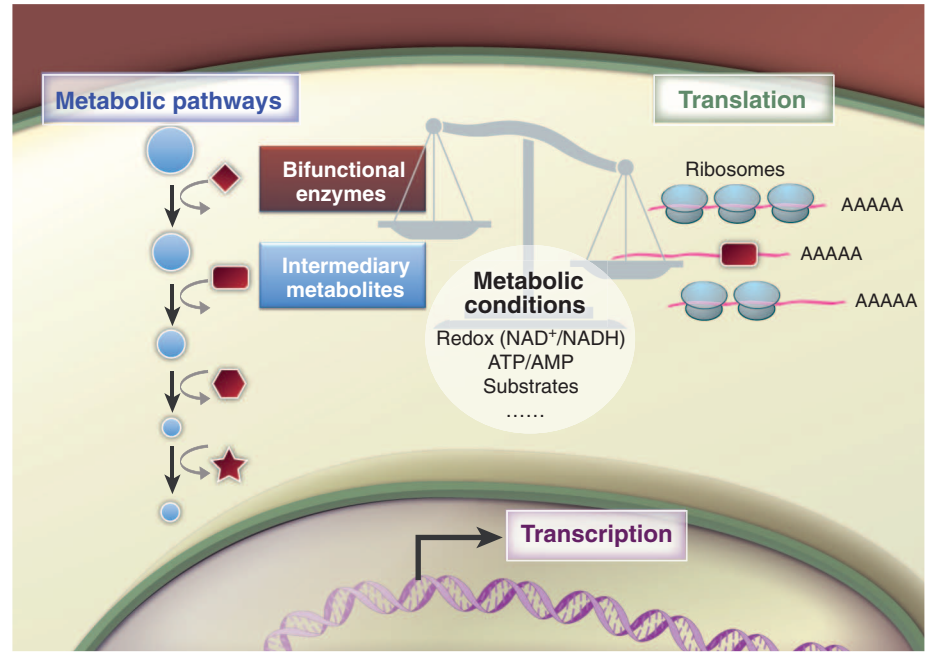


Fig. 5. Bifunctional metabolic enzymes connect metabolism and gene regulation. Metabolic enzymes can moonlight as RNA binding proteins and regulate the translation of specific target mRNAs. The RNA binding function of enzymes can be influenced by interactions with intermediary metabolites and cofactors, leading to posttranscriptional regulation of protein expression. Posttranslational modification of metabolic enzymes could influence their RNA binding function directly or by altering the enzyme's subcellular location. Changes in metabolic conditions, such as bioenergetic demand, hypoxia, stress, and substrate availability, may affect the consequences of the REM interactions. The overall balance of the network between RNA, enzymes, and metabolites can potentially influence T cell fate and function.

cific transcription factors but reinforced by epigenetic modifications on histone tails (H3K4 and H3K27 trimethylation) and DNA methylation of CpG dinucleotides (103). Differential TCA cycle flux or buildup of intermediates such as succinate or fumarate may influence the activity of α -KG-dependent enzymes that regulate T cell epigenetics. This may be one way in which the environment (O_2 , nutrient levels) can influence the plasticity of T_H lineages at sites of infection or inflammation in vivo. Thus, much like the role of oncometabolites in tumorigenesis, studying the metabolism of T cell responses may reveal the existence of “immunometabolites” that influence T cell responses and inflammation.

Connecting Metabolism and Gene Regulation

A transformative conceptual change in the way we consider metabolism within cells is that changes in metabolism can be linked to changes in gene expression through posttranscriptional regulatory networks involving RNA, metabolites, and metabolic enzymes “moonlighting” as RNA binding proteins and regulating specific target mRNAs (104) (Fig. 5). Many enzymes, including those connected with the TCA cycle, glycolysis, PPP, fatty acid metabolism, and other pathways, have been shown to bind RNA in vitro and in cultured cells (105, 106). In addition, the RNA binding function of enzymes can be influenced by interactions with their metabolites or cofactors, illus-

trating how the metabolic state of the cell can control an enzyme's RNA binding function.

The metabolic enzyme perhaps best characterized for its physiological role as an RNA binding protein is cytosolic aconitase (107, 108), a key regulator of cellular iron metabolism (109). In the early 1990s, it was shown that cytosolic aconitase and the RNA binding protein IRP-1 represent the same polypeptide and that the availability of iron triggers insertion or removal of an iron sulfur cluster—switching the protein's function between RNA binding activity (iron low, IRP-1) and metabolic enzyme activity (iron high, aconitase) (110). Remarkably, IRP-1 binds to and regulates mRNAs encoding proteins that function in iron homeostasis. Work from this group and others has led to the idea that bifunctional enzymes and RNA binding proteins may represent a general mechanism of how metabolism and gene expression are coordinated through RNA/enzyme/metabolite (REM) networks [proposed by Hentze and Preiss in (104)].

Lending weight to the REM network hypothesis is a recent study showing that the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), by engaging or disengaging the glycolysis pathway and through fluctuations in its expression, regulates the posttranscriptional production of IFN- γ by T cells (27). GAPDH is a multifunctional enzyme that can bind a range of RNAs, including AU-rich regions in the 3'

untranslated region (UTR) of cytokine mRNAs, including IFN- γ and IL-2 (111). Activated T cells can use either OXPHOS or glycolysis to generate energy to support proliferation and survival. When T cells switch between these ATP-generating programs, as can occur with changes in nutrient availability, or costimulatory or growth factor signals, GAPDH switches from its function as a metabolic enzyme (glycolysis) to its function as an RNA binding protein controlling expression of immunomodulatory factors. When GAPDH is not engaged in glycolysis and OXPHOS is used for ATP production, it binds the 3'UTR of cytokine mRNAs, and translation of these mRNAs is dampened. Thus although OXPHOS can support T cell survival and proliferation, only aerobic glycolysis can facilitate full effector status. This regulatory mechanism provides a checkpoint to allow for uncoupling of T cell proliferation and survival from cytokine production. This is a desirable control mechanism over effector function, because T cells are required to undergo both homeostatic proliferation, when IFN- γ production is neither required nor desirable, and antigen-driven proliferation during an immune response, when effector cytokine production is essential.

The activity of GAPDH is not only controlled by pathway engagement as dictated by substrate availability but is also heavily influenced by redox balance within the cell. For example, GAPDH requires NAD⁺ for its enzymatic function, but NAD⁺ also interferes with mRNA binding, at least in vitro (112). Thus, NAD⁺ controls both enzyme activity and RNA binding in a mutually exclusive fashion. Metabolic enzymes that regulate NAD⁺/NADH balance, including LDHA and PHGDH, may also influence this process. Redox changes may also affect posttranslational modifications of GAPDH, altering its binding to mRNA, metabolites, as well as its localization within the cell (113, 114). This level of regulation between redox balance and RNA binding would be expected to occur with other RNA binding enzymes that share similar dinucleotide binding motifs with GAPDH. Although much work needs to be done to fully understand the biological importance of the interactions between enzymes, RNA, and metabolites, these observations clearly demonstrate how cofactors and substrates generally considered for their direct effects on metabolism may also coordinate metabolism with gene expression.

Future Challenges and Other Considerations

Technical Challenges in Studying Lymphocyte Metabolism

Although interest in studying lymphocyte metabolism and technological advances in metabolomics have increased over the past several years, the field faces many challenges going forward. For instance, although equipment for studying basic metabolism (i.e., oxygen consumption and proton production) is becoming more commonplace, specialized equipment for metabolite measure-

ments [i.e., mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy] and analytical expertise is not routinely accessible to investigators. Another limitation is that, unlike microarray or sequencing technologies, metabolomic analyses do not adhere to a single global platform. For example, gas chromatography coupled to MS (GC-MS) is effective at quantifying organic acids such as TCA cycle intermediates but not most glycolytic intermediates. Thus, multiple platforms must be used to generate complete metabolite data sets. Current extraction methods do not allow for the measurement of subcellular metabolite pools, so information on metabolite localization or channeling between organelles is also lost. One caution of measuring steady-state metabolite levels is that these data provide no measurement of metabolite flux, that is, the speed or direction of metabolite flow through a given pathway (115). Metabolic flux analysis using isotopically labeled metabolites such as ¹³C-glucose or ¹³C-glutamine will be essential for delineating pathways of metabolite flow in T cells.

Perhaps the most relevant challenge for immunologists is the amount of material required for metabolomic analysis. Because of advances in flow cytometry and the identification of new biomarkers to define T cell subsets, immunology has entered an era of cellular subspecialization, where rare cell populations are readily characterized. In comparison, a typical metabolic flux experiment requires millions of cells. This raises the issue of having limited material to analyze the metabolism of cells grown in vitro, let alone in vivo. The development of instrumentation with increased sensitivity will help reduce this gap, but better sample preparation and techniques to amplify metabolite signals are badly needed. The development of genetically encoded fluorescent biosensors for metabolic activity, such as those recently developed for NADH (116) and cellular energy charge (117), will be particularly powerful for studying T cell metabolism at the single-cell level.

Microenvironmental Effects on Metabolism: Are Our Model Systems Correct?

One of the ongoing questions regarding current experimental models in immunology is how closely cell culture models recapitulate immune responses in vivo. From a metabolic perspective, one can conclude that the two systems are worlds apart. Activated T cells cultured in standard medium (Iscove's modified Dulbecco medium plus 10% serum) experience 25 mM glucose (five times standard blood glucose of 5 mM), 4 mM glutamine (eight times the standard concentration of 0.5 mM in blood), and 20% O₂ [two to four times the oxygen tension in blood, which varies depending on tissue type (73)]. Most in vitro assays to assess T cell function are performed at nutrient and O₂ levels much higher than seen in normal physiology; these conditions model a hyperglycemic and hyperoxic environment never seen in vivo.

As T_{EFF} cells move from a nutrient-replete environment in the lymph node or spleen to distant sites of infection, they are likely to experience more restrictive metabolic environments (Fig. 6). Some tissue sites, such as the thymus, bone marrow, and the gastrointestinal epithelium, are naturally hypoxic, whereas inflammation can promote local hypoxic regions in tissues (73). Unlike O₂, which can freely diffuse into tissues, nutrients such as glucose move through space by Brownian motion and require transport into cells, and thus they are likely to have a much more restricted distribution in tissues. Local metabolic activity of immune cells at the site of infection can rapidly consume available O₂ and nutrients, resulting in metabolic stress for infiltrating T cell populations (Fig. 6). There is evidence that T_{EFF} cells in the tumor microenvironment compete with tumor cells for available glucose, and this competition model of nutrient restriction limits the ability of T_{EFF} cells to produce effector cytokines such as IFN- γ (27). Thus, metabolic and environmental influences on T cell function in vivo may elicit very different responses and may account for experimental variance between T cell responses in a petri dish versus what is observed in animal models. Although these points provide sobering food for thought, the development of in vitro methods that control metabolic parameters (e.g., hypoxia incubators, perfusion systems for culture medium) may help to reconcile these differences. Studying metabolite flux of T cells in vivo by using isotopomer labeling techniques will further our understanding of metabolic pathways relevant for T cell function. These techniques are currently being developed in the cancer biology field (118, 119) and should be readily transferable to immunology research.

If activated T cells frequently transition between nutrient-replete states (lymph nodes) and nutrient-deficient states (sites of infection), then management of metabolic resources is an important consideration for lymphocytes in order to ensure maintenance of proliferation and/or effector function. Metabolic insufficiency may be a fundamental mechanism by which environmental context regulates T cell function, potentially influencing T cell tolerance and anergy. Metabolic interference mechanisms, such as indoleamine 2,3-dioxygenase (IDO)-1-dependent degradation of tryptophan by antigen presenting cells (APCs), may represent key regulatory mechanisms at sites of infection or inflammation (120). Tumors may similarly restrict antitumor immunity by influencing T cell metabolism. Competition between tumor cells and tumor-infiltrating T cells for available glucose can impose nutrient deprivation on T_{EFF} cells that limits their ability to produce effector cytokines (27). Tumor-derived lactate can also suppress CTL function directly by blocking lactate export by T cells, thus disrupting their ability to maintain glycolysis (121).

It remains to be determined whether T cells deal with nutrient restriction by displaying metabolic plasticity similar to tumor cells. In this con-

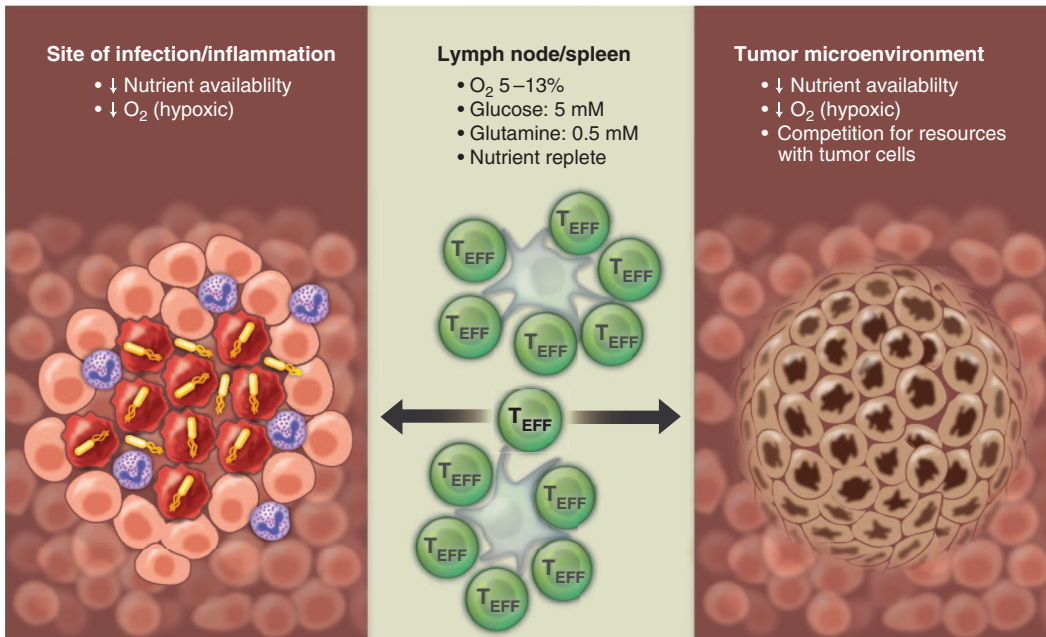


Fig. 6. T cells must display metabolic plasticity to adapt to changes in nutrient and oxygen availability in vivo. T_{EFF} (green) cells must adapt to varying oxygen and nutrient levels depending on environmental context. Lymphoid organs (**middle**) are considered to be nutrient- and oxygen-replete areas, whereas sites of inflammation (**left**) and the tumor microenvironment (**right**) contain hypoxic areas with fluctuations in nutrient availability. At sites of inflammation, nutrient and oxygen availability may become limited because of the metabolic activity of cells at the site of inflammation, necrosis of infected cells, and oxygen consumption by neutrophils. Tumor microenvironments can be highly hypoxic resulting from insufficient vascularization. Additionally, T cells must compete with tumor cells for nutrients such as glucose.

text, AMPK and HIF-1 α , which monitor cellular energy and O_2 levels, respectively, are likely to have a profound effect on the adaptive immune response. Loss of HIF-1 α impairs T_H17 expansion and induction of experimental autoimmune encephalitis (35), consistent with a requirement for HIF-1 α in promoting T_H17 function in vivo. Overall, we know very little regarding pathways that regulate metabolism and adaptation to metabolic stress in lymphocytes, particularly in vivo; this area of immunology is poised for important discoveries in the years to come.

Concluding Thoughts

Recent findings in metabolism and cancer biology have rapidly closed the gap between cell signaling and biochemical pathways. One can now consider all parameters of these fields as being directly intertwined, comprising an interconnected network from gene expression to metabolite production. T cells provide a unique opportunity to understand how metabolism is used in normal biology to achieve proliferation versus abnormal biology, such as that observed in cancer. Characterizing how these pathways are integrated in T cells, how perturbations in the system (i.e., nutrient availability, O_2 tension, and metabolite flux) influence T cell responses, and how metabolic responses are regulated in vivo in the context of infection will be some of the challenges facing scientists in this field. Understanding how environmental cues and cellular metabolism influence the outcome of T cell-

mediated immune responses will continue to be an active area of research in the future. Interfering with metabolic pathways by using agents such as metformin and rapamycin has already revealed substantial and unexpected effects on T cell-mediated immunity. Understanding how metabolic reprogramming influences T cell fate and effector function has the potential to uncover new ways of modulating T cell responses.

References and Notes

1. R. Wang, D. R. Green, Metabolic checkpoints in activated T cells. *Nat. Immunol.* **13**, 907–915 (2012). doi: [10.1038/ni.2386](#); pmid: [22990888](#)
2. E. L. Pearce, E. J. Pearce, Metabolic pathways in immune cell activation and quiescence. *Immunity* **38**, 633–643 (2013). doi: [10.1016/j.immuni.2013.04.005](#); pmid: [23601682](#)
3. L. A. O'Neill, D. G. Hardie, Metabolism of inflammation limited by AMPK and pseudo-starvation. *Nature* **493**, 346–355 (2013). doi: [10.1038/nature11862](#); pmid: [23325217](#)
4. E. F. Greiner, M. Guppy, K. Brand, Glucose is essential for proliferation and the glycolytic enzyme induction that provokes a transition to glycolytic energy production. *J. Biol. Chem.* **269**, 31484–31490 (1994). doi: [10.1074/jbc.269.31484](#); pmid: [7989314](#)
5. M. G. Vander Heiden, L. C. Cantley, C. B. Thompson, Understanding the Warburg effect: The metabolic requirements of cell proliferation. *Science* **324**, 1029–1033 (2009). doi: [10.1126/science.1160809](#); pmid: [19460998](#)
6. K. A. Frauwirth *et al.*, The CD28 signaling pathway regulates glucose metabolism. *Immunity* **16**, 769–777 (2002). doi: [10.1016/S1074-7613\(02\)00323-0](#); pmid: [12121659](#)
7. E. L. Carr *et al.*, Glutamine uptake and metabolism are coordinately regulated by ERK/MAPK during T lymphocyte activation. *J. Immunol.* **185**, 1037–1044 (2010). doi: [10.4049/jimmunol.0903586](#); pmid: [20554958](#)
8. L. V. Sinclair *et al.*, Control of amino-acid transport by antigen receptors coordinates the metabolic reprogramming essential for T cell differentiation. *Nat. Immunol.* **14**, 500–508 (2013). doi: [10.1038/ni.2556](#); pmid: [23525088](#)
9. R. Wang *et al.*, The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. *Immunity* **35**, 871–882 (2011). doi: [10.1016/j.immuni.2011.09.021](#); pmid: [22195744](#)
10. R. D. Michalek *et al.*, Estrogen-related receptor- α is a metabolic regulator of effector T-cell activation and differentiation. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 18348–18353 (2011). doi: [10.1073/pnas.1108856108](#); pmid: [22042850](#)
11. N. J. MacIver, R. D. Michalek, J. C. Rathmell, Metabolic regulation of T lymphocytes. *Annu. Rev. Immunol.* **31**, 259–283 (2013). doi: [10.1146/annurev-immunol-032712-095956](#); pmid: [23298210](#)
12. E. L. Pearce, Metabolism in T cell activation and differentiation. *Curr. Opin. Immunol.* **22**, 314–320 (2010). doi: [10.1016/j.coi.2010.01.018](#); pmid: [20189791](#)
13. G. J. van der Windt *et al.*, Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development. *Immunity* **36**, 68–78 (2012). doi: [10.1016/j.immuni.2011.12.007](#); pmid: [22206904](#)
14. N. Yadava, D. G. Nicholls, Spare respiratory capacity rather than oxidative stress regulates glutamate excitotoxicity after partial respiratory inhibition of mitochondrial complex I with rotenone. *J. Neurosci.* **27**, 7310–7317 (2007). doi: [10.1523/JNEUROSCI.0212-07.2007](#); pmid: [17611283](#)
15. D. G. Nicholls, Spare respiratory capacity, oxidative stress and excitotoxicity. *Biochem. Soc. Trans.* **37**, 1385–1388 (2009). doi: [10.1042/BST0371385](#); pmid: [19909281](#)
16. G. J. van der Windt *et al.*, CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 14336–14341 (2013). doi: [10.1073/pnas.1221740110](#); pmid: [23940348](#)
17. E. L. Pearce *et al.*, Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature* **460**, 103–107 (2009). doi: [10.1038/nature08097](#); pmid: [19494812](#)
18. D. B. Shackelford, R. J. Shaw, The LKB1-AMPK pathway: Metabolism and growth control in tumour suppression. *Nat. Rev. Cancer* **9**, 563–575 (2009). doi: [10.1038/nrc2676](#); pmid: [19629071](#)
19. R. R. Rao, Q. Li, K. Odunsi, P. A. Shrikant, The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin. *Immunity* **32**, 67–78 (2010). doi: [10.1016/j.immuni.2009.10.010](#); pmid: [20060330](#)
20. K. Araki *et al.*, mTOR regulates memory CD8 T-cell differentiation. *Nature* **460**, 108–112 (2009). doi: [10.1038/nature08155](#); pmid: [19543266](#)
21. L. Abu-Elheiga, M. M. Matzuk, K. A. Abo-Hashema, S. J. Wakil, Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2. *Science* **291**, 2613–2616 (2001). doi: [10.1126/science.1056843](#); pmid: [11283375](#)
22. R. J. DeBerardinis, J. J. Lum, C. B. Thompson, Phosphatidylinositol 3-kinase-dependent modulation of carnitine palmitoyltransferase 1A expression regulates lipid metabolism during hematopoietic cell growth. *J. Biol. Chem.* **281**, 37372–37380 (2006). doi: [10.1074/jbc.M608372200](#); pmid: [17030509](#)

23. J. D. McGarry, G. P. Mannaerts, D. W. Foster, A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. *J. Clin. Invest.* **60**, 265–270 (1977). doi: [10.1172/JCI108764](#); pmid: [874089](#)
24. M. Kundu, C. B. Thompson, Autophagy: Basic principles and relevance to disease. *Annu. Rev. Pathol.* **3**, 427–455 (2008). doi: [10.1146/annurev.pathmechdis.2.010506.091842](#); pmid: [18039129](#)
25. V. M. Hubbard *et al.*, Macroautophagy regulates energy metabolism during effector T cell activation. *J. Immunol.* **185**, 7349–7357 (2010). doi: [10.4049/jimmunol.1000576](#); pmid: [21059894](#)
26. H. H. Pua, I. A. Dzhagalov, M. Chuck, N. Mizushima, Y. W. He, A critical role for the autophagy gene Atg5 in T cell survival and proliferation. *J. Exp. Med.* **204**, 25–31 (2007). doi: [10.1084/jem.20061303](#); pmid: [17190837](#)
27. C. H. Chang *et al.*, Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* **153**, 1239–1251 (2013). doi: [10.1016/j.cell.2013.05.016](#); pmid: [23746840](#)
28. G. Chaudhri, I. A. Clark, N. H. Hunt, W. B. Cowden, R. Ceredig, Effect of antioxidants on primary alloantigen-induced T cell activation and proliferation. *J. Immunol.* **137**, 2646–2652 (1986). pmid: [2944959](#)
29. S. Devadas, L. Zaritskaya, S. G. Rhee, L. Oberley, M. S. Williams, Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: Selective regulation of mitogen-activated protein kinase activation and fas ligand expression. *J. Exp. Med.* **195**, 59–70 (2002). doi: [10.1084/jem.20010659](#); pmid: [11781366](#)
30. S. H. Jackson, S. Devadas, J. Kwon, L. A. Pinto, M. S. Williams, T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation. *Nat. Immunol.* **5**, 818–827 (2004). doi: [10.1038/n1096](#); pmid: [15258578](#)
31. L. A. Sena *et al.*, Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling. *Immunity* **38**, 225–236 (2013). doi: [10.1016/j.immuni.2012.10.020](#); pmid: [23415911](#)
32. S. Krauss, M. D. Brand, F. Buttgerit, Signaling takes a breath—New quantitative perspectives on bioenergetics and signal transduction. *Immunity* **15**, 497–502 (2001). doi: [10.1016/S1074-7613\(01\)00205-9](#); pmid: [11672532](#)
33. R. G. Jones *et al.*, The proapoptotic factors Bax and Bak regulate T Cell proliferation through control of endoplasmic reticulum Ca(2+) homeostasis. *Immunity* **27**, 268–280 (2007). doi: [10.1016/j.immuni.2007.05.023](#); pmid: [17692540](#)
34. R. D. Michalek *et al.*, Cutting edge: Distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. *J. Immunol.* **186**, 3299–3303 (2011). doi: [10.4049/jimmunol.1003613](#); pmid: [21317389](#)
35. L. Z. Shi *et al.*, HIF1 α -dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of T_H17 and T_{Reg} cells. *J. Exp. Med.* **208**, 1367–1376 (2011). doi: [10.1084/jem.20110278](#); pmid: [21708926](#)
36. E. V. Dang *et al.*, Control of T(H)17/T(Reg) balance by hypoxia-inducible factor 1. *Cell* **146**, 772–784 (2011). doi: [10.1016/j.cell.2011.07.033](#); pmid: [21871655](#)
37. C. Wu *et al.*, Induction of pathogenic T_H17 cells by inducible salt-sensing kinase SGK1. *Nature* **496**, 513–517 (2013). doi: [10.1038/nature11984](#); pmid: [23467085](#)
38. M. Kleinnietfeld *et al.*, Sodium chloride drives autoimmune disease by the induction of pathogenic T_H17 cells. *Nature* **496**, 518–522 (2013). doi: [10.1038/nature11868](#); pmid: [23467095](#)
39. P. M. Smith *et al.*, The microbial metabolites, short-chain fatty acids, regulate colonic T_{Reg} cell homeostasis. *Science* **341**, 569–573 (2013). doi: [10.1126/science.1241165](#). doi: [10.1126/science.1241165](#); pmid: [23828891](#)
40. P. S. Ward, C. B. Thompson, Metabolic reprogramming: A cancer hallmark even Warburg did not anticipate. *Cancer Cell* **21**, 297–308 (2012). doi: [10.1016/j.ccr.2012.02.014](#); pmid: [22439925](#)
41. C. A. Doughty *et al.*, Antigen receptor-mediated changes in glucose metabolism in B lymphocytes: Role of phosphatidylinositol 3-kinase signaling in the glycolytic control of growth. *Blood* **107**, 4458–4465 (2006). doi: [10.1182/blood-2005-12-4788](#); pmid: [16449529](#)
42. C. M. Krawczyk *et al.*, Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood* **115**, 4742–4749 (2010). doi: [10.1182/blood-2009-10-249540](#); pmid: [20351312](#)
43. A. Haschemi *et al.*, The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. *Cell Metab.* **15**, 813–826 (2012). doi: [10.1016/j.cmet.2012.04.023](#); pmid: [22682222](#)
44. R. J. DeBerardinis, N. Sayed, D. Ditsworth, C. B. Thompson, Brick by brick: Metabolism and tumor cell growth. *Curr. Opin. Genet. Dev.* **18**, 54–61 (2008). doi: [10.1016/j.gde.2008.02.003](#); pmid: [18387799](#)
45. K. E. Wellen, C. B. Thompson, A two-way street: Reciprocal regulation of metabolism and signalling. *Nat. Rev. Mol. Cell Biol.* **13**, 270–276 (2012). pmid: [2295772](#)
46. R. J. DeBerardinis, J. J. Lum, G. Hatzivassiliou, C. B. Thompson, The biology of cancer: Metabolic reprogramming fuels cell growth and proliferation. *Cell Metab.* **7**, 11–20 (2008). doi: [10.1016/j.cmet.2007.10.002](#); pmid: [18177721](#)
47. G. Hatzivassiliou *et al.*, ATP citrate lyase inhibition can suppress tumor cell growth. *Cancer Cell* **8**, 311–321 (2005). doi: [10.1016/j.ccr.2005.09.008](#); pmid: [16226706](#)
48. Y. Kidani *et al.*, Sterol regulatory element-binding proteins are essential for the metabolic programming of effector T cells and adaptive immunity. *Nat. Immunol.* **14**, 489–499 (2013). doi: [10.1038/ni.2570](#); pmid: [23563690](#)
49. Q. Wang *et al.*, Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. *Science* **327**, 1004–1007 (2010). doi: [10.1126/science.1179687](#); pmid: [20167787](#)
50. S. Zhao *et al.*, Regulation of cellular metabolism by protein lysine acetylation. *Science* **327**, 1000–1004 (2010). doi: [10.1126/science.1179689](#); pmid: [20167786](#)
51. K. E. Wellen *et al.*, ATP-citrate lyase links cellular metabolism to histone acetylation. *Science* **324**, 1076–1080 (2009). doi: [10.1126/science.1164097](#); pmid: [19461003](#)
52. M. Griffiths, D. Keast, The effect of glutamine on murine splenic leukocyte responses to T and B cell mitogens. *Immunol. Cell Biol.* **68**, 405–408 (1990). doi: [10.1038/icb.1990.54](#); pmid: [2097296](#)
53. A. Le *et al.*, Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. *Cell Metab.* **15**, 110–121 (2012). doi: [10.1016/j.cmet.2011.12.009](#); pmid: [2225880](#)
54. S. Mazurek, Pyruvate kinase type M2: A key regulator of the metabolic budget system in tumor cells. *Int. J. Biochem. Cell Biol.* **43**, 969–980 (2011). doi: [10.1016/j.jbiocel.2010.02.005](#); pmid: [20156581](#)
55. S. Marjanovic, I. Eriksson, B. D. Nelson, Expression of a new set of glycolytic isozymes in activated human peripheral lymphocytes. *Biochim. Biophys. Acta* **1087**, 1–6 (1990). doi: [10.1016/0167-4781\(90\)90113-G](#); pmid: [2169315](#)
56. D. Anastasiou *et al.*, Pyruvate kinase M2 activators promote tetramer formation and suppress tumorigenesis. *Nat. Chem. Biol.* **8**, 839–847 (2012). doi: [10.1038/nchembio.1060](#); pmid: [22922757](#)
57. H. R. Christofk *et al.*, The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* **452**, 230–233 (2008). doi: [10.1038/nature06734](#); pmid: [18337823](#)
58. H. R. Christofk, M. G. Vander Heiden, N. Wu, J. M. Asara, L. C. Cantley, Pyruvate kinase M2 is a phosphotyrosine-binding protein. *Nature* **452**, 181–186 (2008). doi: [10.1038/nature06667](#); pmid: [18337815](#)
59. T. Hitosugi *et al.*, Tyrosine phosphorylation inhibits PKM2 to promote the Warburg effect and tumor growth. *Sci. Signal.* **2**, ra73 (2009). doi: [10.1126/scisignal.2000431](#); pmid: [19920251](#)
60. C. Kung *et al.*, Small molecule activation of PKM2 in cancer cells induces serine auxotrophy. *Chem. Biol.* **19**, 1187–1198 (2012). doi: [10.1016/j.chembiol.2012.07.021](#); pmid: [22999886](#)
61. W. Luo *et al.*, Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. *Cell* **145**, 732–744 (2011). doi: [10.1016/j.cell.2011.03.054](#); pmid: [21620138](#)
62. W. Yang *et al.*, PKM2 phosphorylates histone H3 and promotes gene transcription and tumorigenesis. *Cell* **150**, 685–696 (2012). doi: [10.1016/j.cell.2012.07.018](#); pmid: [22901803](#)
63. X. Gao, H. Wang, J. J. Yang, X. Liu, Z. R. Liu, Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase. *Mol. Cell* **45**, 598–609 (2012). doi: [10.1016/j.molcel.2012.01.001](#); pmid: [22306293](#)
64. B. Chaneton *et al.*, Serine is a natural ligand and allosteric activator of pyruvate kinase M2. *Nature* **491**, 458–462 (2012). doi: [10.1038/nature11540](#); pmid: [23064226](#)
65. J. W. Locasale *et al.*, Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis. *Nat. Genet.* **43**, 869–874 (2011). doi: [10.1038/ng.890](#); pmid: [21804546](#)
66. R. Possemato *et al.*, Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. *Nature* **476**, 346–350 (2011). doi: [10.1038/nature10350](#); pmid: [21760589](#)
67. O. D. Maddocks *et al.*, Serine starvation induces stress and p53-dependent metabolic remodelling in cancer cells. *Nature* **493**, 542–546 (2013). doi: [10.1038/nature11743](#); pmid: [23242140](#)
68. L. M. Nilsson *et al.*, Mouse genetics suggests cell-context dependency for Myc-regulated metabolic enzymes during tumorigenesis. *PLoS Genet.* **8**, e1002573 (2012). doi: [10.1371/journal.pgen.1002573](#); pmid: [22438825](#)
69. R. J. DeBerardinis *et al.*, Beyond aerobic glycolysis: Transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 19345–19350 (2007). doi: [10.1073/pnas.0709747104](#); pmid: [18032601](#)
70. A. R. Mullen *et al.*, Reductive carboxylation supports growth in tumour cells with defective mitochondria. *Nature* **481**, 385–388 (2012). pmid: [22101431](#)
71. C. M. Metallo *et al.*, Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature* **481**, 380–384 (2012). pmid: [22101433](#)
72. D. R. Wise *et al.*, Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of α -ketoglutarate to citrate to support cell growth and viability. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 19611–19616 (2011). doi: [10.1073/pnas.111773108](#); pmid: [22106302](#)
73. E. N. McNamee, D. Korn Johnson, D. Homann, E. T. Clambey, Hypoxia and hypoxia-inducible factors as regulators of T cell development, differentiation, and function. *Immunol. Res.* **55**, 58–70 (2013). doi: [10.1007/s12026-012-8349-8](#); pmid: [22961658](#)
74. H. K. Eltzschig, P. Carmeliet, Hypoxia and inflammation. *N. Engl. J. Med.* **364**, 656–665 (2011). doi: [10.1056/NEJMra0910283](#); pmid: [21323543](#)
75. H. Nakamura *et al.*, TCR engagement increases hypoxia-inducible factor-1 α protein synthesis via rapamycin-sensitive pathway under hypoxic conditions in human peripheral T cells. *J. Immunol.* **174**, 7592–7599 (2005). pmid: [15944259](#)
76. E. T. Clambey *et al.*, Hypoxia-inducible factor-1 α -dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa. *Proc. Natl. Acad. Sci. U.S.A.* **109**, E2784–E2793 (2012). doi: [10.1073/pnas.1202366109](#); pmid: [22988108](#)
77. D. W. Parsons *et al.*, An integrated genomic analysis of human glioblastoma multiforme. *Science* **321**, 1807–1812 (2008). doi: [10.1126/science.1164382](#). doi: [10.1126/science.1164382](#); pmid: [18772396](#)
78. L. Dang *et al.*, Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* **462**, 739–744 (2009). doi: [10.1038/nature08617](#); pmid: [19935646](#)
79. M. E. Figueroa *et al.*, Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2

- function, and impair hematopoietic differentiation. *Cancer Cell* **18**, 553–567 (2010). doi: [10.1016/j.ccr.2010.11.015](#); pmid: [21130701](#)
80. C. Lu *et al.*, IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature* **483**, 474–478 (2012). doi: [10.1038/nature10860](#); pmid: [22343901](#)
 81. W. Xu *et al.*, Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α -ketoglutarate-dependent dioxygenases. *Cancer Cell* **19**, 17–30 (2011). doi: [10.1016/j.ccr.2010.12.014](#); pmid: [21251613](#)
 82. S. Turcan *et al.*, IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature* **483**, 479–483 (2012). doi: [10.1038/nature10866](#); pmid: [22343889](#)
 83. D. G. Hardie, F. A. Ross, S. A. Hawley, AMPK: A nutrient and energy sensor that maintains energy homeostasis. *Nat. Rev. Mol. Cell Biol.* **13**, 251–262 (2012). doi: [10.1038/nrm3311](#); pmid: [22436748](#)
 84. B. Xiao *et al.*, Structure of mammalian AMPK and its regulation by ADP. *Nature* **472**, 230–233 (2011). doi: [10.1038/nature09932](#); pmid: [21399626](#)
 85. J. S. Oakhill *et al.*, AMPK is a direct adenylate charge-regulated protein kinase. *Science* **332**, 1433–1435 (2011). doi: [10.1126/science.1200094](#); pmid: [21680840](#)
 86. F. Buttgerit, M. D. Brand, A hierarchy of ATP-consuming processes in mammalian cells. *Biochem. J.* **312**, 163–167 (1995). pmid: [7492307](#)
 87. P. Tamás *et al.*, Regulation of the energy sensor AMP-activated protein kinase by antigen receptor and Ca²⁺ in T lymphocytes. *J. Exp. Med.* **203**, 1665–1670 (2006). doi: [10.1084/jem.20052469](#); pmid: [16818670](#)
 88. N. J. MacIver *et al.*, The liver kinase B1 is a central regulator of T cell development, activation, and metabolism. *J. Immunol.* **187**, 4187–4198 (2011). doi: [10.4049/jimmunol.1100367](#); pmid: [21930968](#)
 89. B. Faubert *et al.*, AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo. *Cell Metab.* **17**, 113–124 (2013). doi: [10.1016/j.cmet.2012.12.001](#); pmid: [23274086](#)
 90. P. Tamás *et al.*, LKB1 is essential for the proliferation of T-cell progenitors and mature peripheral T cells. *Eur. J. Immunol.* **40**, 242–253 (2010). doi: [10.1002/eji.200939677](#); pmid: [19830737](#)
 91. R. G. Jones *et al.*, AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol. Cell* **18**, 283–293 (2005). doi: [10.1016/j.molcel.2005.03.027](#); pmid: [15866171](#)
 92. J. Rolf *et al.*, AMPK α 1: A glucose sensor that controls CD8 T-cell memory. *Eur. J. Immunol.* **43**, 889–896 (2013). doi: [10.1002/eji.201243008](#); pmid: [23310952](#)
 93. N. L. Alderson *et al.*, S-(2-Succinyl)cytosine: A novel chemical modification of tissue proteins by a Krebs cycle intermediate. *Arch. Biochem. Biophys.* **450**, 1–8 (2006). doi: [10.1016/j.abb.2006.03.005](#); pmid: [16624247](#)
 94. C. Bardella *et al.*, Aberrant succination of proteins in fumarate hydratase-deficient mice and HLRCC patients is a robust biomarker of mutation status. *J. Pathol.* **225**, 4–11 (2011). doi: [10.1002/path.2932](#); pmid: [21630274](#)
 95. J. Adam *et al.*, Renal cyst formation in Fh1-deficient mice is independent of the Hif/Phd pathway: Roles for fumarate in KEAP1 succination and Nrf2 signaling. *Cancer Cell* **20**, 524–537 (2011). doi: [10.1016/j.ccr.2011.09.006](#); pmid: [22014577](#)
 96. W. G. Kaelin Jr., Cancer and altered metabolism: Potential importance of hypoxia-inducible factor and 2-oxoglutarate-dependent dioxygenases. *Cold Spring Harb. Symp. Quant. Biol.* **76**, 335–345 (2011). doi: [10.1101/sqb.2011.76.010975](#); pmid: [22089927](#)
 97. G. L. Semenza, Hypoxia-inducible factors in physiology and medicine. *Cell* **148**, 399–408 (2012). doi: [10.1016/j.cell.2012.01.021](#); pmid: [22304911](#)
 98. J. A. Losman, W. G. Kaelin Jr., What a difference a hydroxyl makes: Mutant IDH, (R)-2-hydroxyglutarate, and cancer. *Genes Dev.* **27**, 836–852 (2013). doi: [10.1101/gad.217406.113](#); pmid: [23630074](#)
 99. P. S. Ward *et al.*, The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting α -ketoglutarate to 2-hydroxyglutarate. *Cancer Cell* **17**, 225–234 (2010). doi: [10.1016/j.ccr.2010.01.020](#); pmid: [20171147](#)
 100. J. S. Isaacs *et al.*, HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: Novel role of fumarate in regulation of HIF stability. *Cancer Cell* **8**, 143–153 (2005). doi: [10.1016/j.ccr.2005.06.017](#); pmid: [16098467](#)
 101. M. A. Selak *et al.*, Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF- α prolyl hydroxylase. *Cancer Cell* **7**, 77–85 (2005). doi: [10.1016/j.ccr.2004.11.022](#); pmid: [15652751](#)
 102. G. M. Tannahill *et al.*, Succinate is an inflammatory signal that induces IL-1 β through HIF-1 α . *Nature* **496**, 238–242 (2013). doi: [10.1038/nature11986](#); pmid: [23535595](#)
 103. Y. Kanno, G. Vahedi, K. Hirahara, K. Singleton, J. J. O'Shea, Transcriptional and epigenetic control of T helper cell specification: Molecular mechanisms underlying commitment and plasticity. *Annu. Rev. Immunol.* **30**, 707–731 (2012). doi: [10.1146/annurev-immunol-020711-075058](#); pmid: [22224760](#)
 104. M. W. Hentze, T. Preiss, The REM phase of gene regulation. *Trends Biochem. Sci.* **35**, 423–426 (2010). doi: [10.1016/j.tibs.2010.05.009](#); pmid: [20554447](#)
 105. A. Castello *et al.*, Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. *Cell* **149**, 1393–1406 (2012). doi: [10.1016/j.cell.2012.04.031](#); pmid: [22658674](#)
 106. J. Cieřla, Metabolic enzymes that bind RNA: Yet another level of cellular regulatory network? *Acta Biochim. Pol.* **53**, 11–32 (2006). pmid: [16410835](#)
 107. M. W. Hentze, P. Argos, Homology between IRE-BP, a regulatory RNA-binding protein, aconitase, and isopropylmalate isomerase. *Nucleic Acids Res.* **19**, 1739–1740 (1991). doi: [10.1093/nar/19.8.1739](#); pmid: [1903202](#)
 108. T. A. Rouault, C. D. Stout, S. Kaptain, J. B. Harford, R. D. Klausner, Structural relationship between an iron-regulated RNA-binding protein (IRE-BP) and aconitase: Functional implications. *Cell* **64**, 881–883 (1991). doi: [10.1016/0092-8674\(91\)90312-M](#); pmid: [2001588](#)
 109. M. W. Hentze, M. U. Muckenthaler, B. Galy, C. Camaschella, Two to tango: Regulation of Mammalian iron metabolism. *Cell* **142**, 24–38 (2010). doi: [10.1016/j.cell.2010.06.028](#); pmid: [20603012](#)
 110. A. Constable, S. Quick, N. K. Gray, M. W. Hentze, Modulation of the RNA-binding activity of a regulatory protein by iron in vitro: Switching between enzymatic and genetic function? *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4554–4558 (1992). doi: [10.1073/pnas.89.10.4554](#); pmid: [1584791](#)
 111. E. Nagy, W. F. C. Rigby, Glyceraldehyde-3-phosphate dehydrogenase selectively binds AU-rich RNA in the NAD (+)-binding region (Rossmann fold). *J. Biol. Chem.* **270**, 2755–2763 (1995). doi: [10.1074/jbc.270.6.2755](#); pmid: [7531693](#)
 112. E. Nagy *et al.*, Identification of the NAD(+)-binding fold of glyceraldehyde-3-phosphate dehydrogenase as a novel RNA-binding domain. *Biochem. Biophys. Res. Commun.* **275**, 253–260 (2000). doi: [10.1006/bbrc.2000.3246](#); pmid: [10964654](#)
 113. A. Colell *et al.*, GAPDH and autophagy preserve survival after apoptotic cytochrome c release in the absence of caspase activation. *Cell* **129**, 983–997 (2007). doi: [10.1016/j.cell.2007.03.045](#); pmid: [17540177](#)
 114. C. Tristan, N. Shahani, T. W. Sedlak, A. Sawa, The diverse functions of GAPDH: Views from different subcellular compartments. *Cell. Signal.* **23**, 317–323 (2011). doi: [10.1016/j.cellsig.2010.08.003](#); pmid: [20727968](#)
 115. R. J. DeBerardinis, C. B. Thompson, Cellular metabolism and disease: What do metabolic outliers teach us? *Cell* **148**, 1132–1144 (2012). doi: [10.1016/j.cell.2012.02.032](#); pmid: [22424225](#)
 116. Y. P. Hung, J. G. Albeck, M. Tantama, G. Yellen, Imaging cytosolic NADH-NAD(+) redox state with a genetically encoded fluorescent biosensor. *Cell Metab.* **14**, 545–554 (2011). doi: [10.1016/j.cmet.2011.08.012](#); pmid: [21982714](#)
 117. P. Tsou, B. Zheng, C. H. Hsu, A. T. Sasaki, L. C. Cantley, A fluorescent reporter of AMPK activity and cellular energy stress. *Cell Metab.* **13**, 476–486 (2011). doi: [10.1016/j.cmet.2011.03.006](#); pmid: [21459332](#)
 118. I. Marín-Valencia *et al.*, Analysis of tumor metabolism reveals mitochondrial glucose oxidation in genetically diverse human glioblastomas in the mouse brain in vivo. *Cell Metab.* **15**, 827–837 (2012). doi: [10.1016/j.cmet.2012.05.001](#); pmid: [22682223](#)
 119. I. Marín-Valencia *et al.*, Glucose metabolism via the pentose phosphate pathway, glycolysis and Krebs cycle in an orthotopic mouse model of human brain tumors. *NMR Biomed.* **25**, 1177–1186 (2012). doi: [10.1002/nbm.2787](#); pmid: [22383401](#)
 120. A. L. Mellor, D. H. Munn, IDO expression by dendritic cells: Tolerance and tryptophan catabolism. *Nat. Rev. Immunol.* **4**, 762–774 (2004). doi: [10.1038/nri1457](#); pmid: [15459668](#)
 121. K. Fischer *et al.*, Inhibitory effect of tumor cell-derived lactic acid on human T cells. *Blood* **109**, 3812–3819 (2007). doi: [10.1182/blood-2006-07-035972](#); pmid: [17255361](#)

Acknowledgments: We thank J. Blagih, E. Clambey, M. Hentze, C. Krawczyk, M. Vander Heiden, and members of the Jones and Pearce laboratories for insight and comments on this manuscript and L. Donnelly and M. Maslowska for administrative help. This work was supported by grants to R.G.J. from the Canadian Institute for Health Research (MOP-93799 and a New Investigator Career Award) and the Arthritis Society of Canada and by grants to E.L.P. from National Institute of Allergy and Infectious Diseases (AI091965) and National Cancer Institute (CA158823).

Opposite Feedbacks in the Hippo Pathway for Growth Control and Neural Fate

David Jukam,* Baotong Xie,* Jens Rister,* David Terrell, Mark Charlton-Perkins, Daniela Pistillo, Brian Gebelein, Claude Desplan,[†] Tiffany Cook[†]

READ THE FULL ARTICLE ONLINE

<http://dx.doi.org/10.1126/science.1238016>



Cite this article as D. Jukam *et al.*, *Science* **342**, 1238016 (2013). DOI: 10.1126/science.1238016

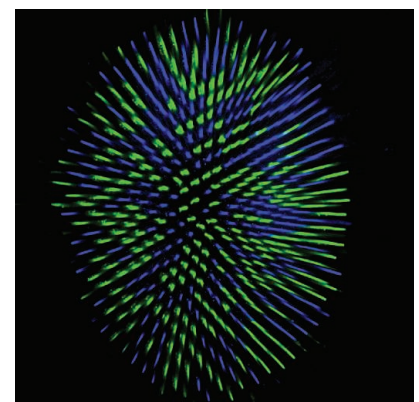
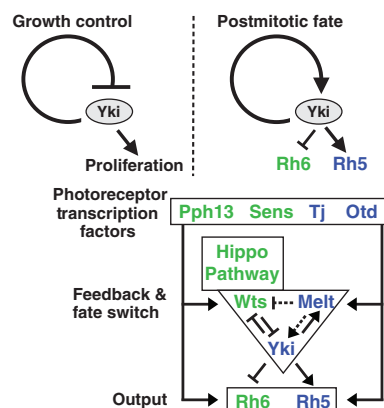
Introduction: A finite number of signaling pathways are repurposed during animal development to regulate an extraordinary array of cellular decisions. Elucidating context-specific mechanisms is crucial for understanding how cellular diversity is generated and for defining potential avenues of pathway misregulation during disease. The Hippo tumor suppressor pathway has been primarily studied in growth control where it inhibits the oncogenic transcriptional coactivator Yorkie (Yki) (YAP/TAZ in vertebrates). The Hippo pathway also functions in nongrowth contexts such as postmitotic fate specification. In the *Drosophila* visual system, R8 photoreceptor neurons terminally differentiate into one of two alternative subtypes that express either blue-light-sensitive Rhodopsin5 (Rh5) or green-light-sensitive Rhodopsin6 (Rh6). These mutually exclusive cell fates are established by the Hippo pathway kinase *warts* and the growth regulator gene *meltd*, which repress each other's expression. However, the mechanisms underlying the context-specific use of the Hippo pathway in postmitotic fate decisions remain unclear.

Methods: To define the regulatory mechanisms of Hippo-dependent cell fate decisions in *Drosophila* photoreceptor neurons, we used a combination of genetic epistasis analyses, in vivo cis-regulatory studies, a candidate gene RNA interference screen, and cell culture-based transcription assays.

Results: We show that the transcriptional output of the Hippo pathway in photoreceptor differentiation, as in cell proliferation, is mediated through the factors Yki and Scalloped. In contrast to growth control, where Yki limits its own activity by negative feedback, we identify two Yki positive-feedback mechanisms: In blue-sensitive Rh5 photoreceptors, Yki represses its own negative regulator *warts*, downstream of *meltd*; Yki also promotes *meltd* expression, which subsequently represses *warts* to further promote Yki function. Yki cooperates with the transcription factors Orthodenticle (Otd) and Traffic Jam (Tj) to promote *meltd* expression and Rh5 photoreceptor fate. Otd and Tj, orthologs of the mammalian OTX/CRX and MAF/NRL transcription factors, form an evolutionarily conserved transcriptional module for generating photoreceptor subtype diversity. We also show that the transcription factors Senseless and Pph13 create a permissive environment for Warts/Hippo signaling to promote the alternative "default" green-sensitive Rh6 fate. Hence, Hippo pathway function integrates with four cell-type-restricted transcription factors, each promoting genetically different aspects of R8 subtypes, such that Yki activity ultimately coordinates the binary fate decision between blue- and green-sensitive photoreceptors.

Discussion: This work illustrates how molecular signaling pathways can adopt context-specific regulation. Yki positive feedback in the photoreceptor fate decision is opposite to the negative feedback found in Hippo growth control. These distinct network-level feedback mechanisms provide context-appropriate functions: homeostasis to fine-tune growth regulation and an all-or-nothing fate decision to ensure robust differentiation of sensory neuron subtypes. Altering network-level systems properties, such as positive or negative feedback, within biochemically conserved pathways may be broadly used to co-opt signaling networks for use in cellular contexts as distinct as proliferation and terminal differentiation.

Context-specific regulation by the Hippo signaling in postmitotic photoreceptors. The Hippo pathway uses negative feedback through its transcriptional effector Yki for homeostatic control of proliferation. In *Drosophila* eyes, two alternative fates of blue- versus green-sensitive R8 photoreceptors are regulated by antagonism between the growth regulator *Meltd* and the Hippo pathway. Contrary to the growth mechanism, Yki positive feedback and a cell-type-restricted transcription factor network promote repurposing of the Hippo pathway for binary fate decisions.



FIGURES IN THE FULL ARTICLE

Fig. 1. Yki and Sd instruct mutually exclusive R8 neuron subtypes.

Fig. 2. Yki and Sd regulate *wts* and *melt* expression in Hippo pathway positive feedback.

Fig. 3. The photoreceptor regulator Otd promotes *melt* expression in pR8s.

Fig. 4. Tissue-restricted transcription factors control *melt* and *Rhodopsins* with regulatory logic conserved in mammalian eye development.

Fig. 5. The Hippo pathway requires photoreceptor specification factors to regulate R8 subtypes.

Fig. 6. Tissue-restricted transcription factors promote reuse of the Hippo pathway with positive feedback for postmitotic neuronal fate.

SUPPLEMENTARY MATERIALS

Materials and Methods

Figs. S1 to S13

References (46–65)

The list of author affiliations is available in the full article online. *These authors contributed equally to this work.

[†]Corresponding author. E-mail: tiffany.cook@cchmc.org (T.C.); claude.desplan@nyu.edu (C.D.)

Opposite Feedbacks in the Hippo Pathway for Growth Control and Neural Fate

David Jukam,^{1*†} Baotong Xie,^{2*‡} Jens Rister,^{1*} David Terrell,² Mark Charlton-Perkins,² Daniela Pistillo,¹ Brian Gebelein,³ Claude Desplan,^{1§} Tiffany Cook^{2,3§}

Signaling pathways are reused for multiple purposes in plant and animal development. The Hippo pathway in mammals and *Drosophila* coordinates proliferation and apoptosis via the coactivator and oncoprotein YAP/Yorkie (Yki), which is homeostatically regulated through negative feedback. In the *Drosophila* eye, cross-repression between the Hippo pathway kinase LATS/Warts (Wts) and growth regulator Melted generates mutually exclusive photoreceptor subtypes. Here, we show that this all-or-nothing neuronal differentiation results from Hippo pathway positive feedback: Yki both represses its negative regulator, *warts*, and promotes its positive regulator, *melted*. This postmitotic Hippo network behavior relies on a tissue-restricted transcription factor network—including a conserved Otx/Orthodenticle-Nrl/Traffic Jam feedforward module—that allows Warts-Yki-Melted to operate as a bistable switch. Altering feedback architecture provides an efficient mechanism to co-opt conserved signaling networks for diverse purposes in development and evolution.

Core signaling pathways are reused for different purposes during development, allowing extraordinary cell-type diversity (1). For example, the transforming growth factor- β (TGF- β), Notch, receptor tyrosine kinase/mitogen-activated protein kinase (RTK/MAPK), and Wnt signaling pathways each act repeatedly, from embryogenesis to adulthood, to coordinate tissue patterning, growth, and specification throughout the animal. The Hippo pathway is best known for its role in growth control in both flies and mammals, where it regulates the balance between division and death in mitotic cells (2). But the Hippo pathway also regulates postmitotic events such as photoreceptor subtype specification in the *Drosophila* eye (3, 4). How the same signaling network can be regulated for context-appropriate outcomes as diverse as proliferation and differentiation is not well understood.

The *Drosophila* eye comprises about 800 unit eyes (ommatidia), each containing eight photoreceptors (R1 to R8) (5). Two main ommatidial subtypes are defined by light-sensing Rhodopsin (Rh) proteins expressed in the color vision photoreceptors R7 and R8: “p” ommatidia, with ultraviolet (UV)-sensitive Rh3 in R7 and blue-Rh5 in R8, and “y” ommatidia with longer UV-Rh4 in R7 and green-Rh6 in R8 (Fig. 1A) [reviewed

in (6)]. p and y subtypes are distributed randomly in the retina in a p:y ratio of ~30:70, following stochastic expression of the transcription factor Spineless in the R7 of subtype y (yR7s). pR7s, which lack Spineless, signal to underlying R8s to induce pR8/Rh5 fate, whereas the remaining R8s become yR8/Rh6 by default (6). p versus y fate in R8s is established by a bistable transcriptional feedback loop between Melted (Melt), a pleckstrin homology-domain protein that specifies pR8/Rh5 fate, and Wts, a kinase in the Hippo pathway that specifies yR8/Rh6 fate (Fig. 1, B and C) (3).

In its canonical role as a tumor suppressor, Wts is activated by the Hippo kinase (Hpo) and phosphorylates Yki, the *Drosophila* ortholog of the human oncoprotein YAP, to sequester Yki in the cytoplasm (2). In the absence of Hippo signaling, nonphosphorylated Yki enters the nucleus and binds as a coactivator to transcription factors like Scalloped (Sd), Homothorax (Hth), and Mothers against Dpp (Mad) (7–11) to activate target proliferation and anti-apoptotic genes. Yki can also induce its negative regulators *expanded*, *merlin*, *kibra*, or *dmyc* to provide negative feedback onto itself during growth control (12–14).

Here we show that in postmitotic R8s, as in growth, Yki and its DNA-binding partner Sd mediate transcriptional output of the Hippo pathway. However, the R8 regulatory architecture is fundamentally different, as Yki promotes positive feedback onto itself. This regulation requires a tissue-specific transcription factor network that includes Orthodenticle (Otd) and Traffic Jam (Tj), orthologs of the mammalian photoreceptor determinants Crx and Nrl (15), respectively, as well as Pph13 and Gfil1/Senseless (Sens). This network generates the postmitotic context for the Hippo pathway to regulate an all-or-nothing fate decision and ensure robust terminal differentiation of sensory neuron subtypes.

Yki and Sd Regulate R8 Subtype Specification

To test whether Yki functions in R8 neurons, we manipulated *yki* and assayed Rh5 and Rh6 expression. *yki* null mutant eye progenitor cells do not divide and are eliminated by apoptosis (16). We therefore used Gal4 drivers to express *yki*-targeted RNA interference (RNAi) in postmitotic photoreceptors. Knockdown of *yki* in all photoreceptors throughout development (*IGMR>yki^{RNAi}*), only in adults (using Gal80^{ts}), or in all R8s (and some R1 to R6) (*sens>yki^{RNAi}*) caused almost all R8s to express Rh6, whereas Rh5 was nearly absent (Fig. 1D and fig. S1, A and C). Conversely, overexpressing wild-type or activated *yki*/YAP (*yki^{S168A}* or human *YAP^{S127A}*) (17, 18) in all photoreceptors (*IGMR>yki*) transformed almost all R8s into Rh5-expressing pR8s (Fig. 1D and fig. S5C). Ectopic Yki did not require the pR7 signal to induce pR8 fate because Yki induced Rh5 even in the absence of R7s (*sev*; *GMR>yki*) (fig. S2A). Furthermore, misexpressing *yki* only in yR8s after the fate decision (*Rh6>yki*) also induced Rh5 (fig. S2C). *yki* manipulations did not affect general neuronal fate, specific photoreceptor fate, or expression of other Rhodopsins (fig. S2, B, D, E, and F). Thus, *yki* is necessary and sufficient in R8s to specify pR8/Rh5 and prevent yR8/Rh6 subtypes.

Yki is a cofactor for DNA-binding transcription factors such as Sd, Hth, or Mad (7–11) to activate Hippo target genes. *hth* or *mad* loss-of-function [*IGMR>hth^{DN}* (19) and *IGMR>ey>mad^{RNAi}*] (fig. S3D) did not noticeably affect Rh5 or Rh6. In contrast, retinas with *sd* loss-of-function (*sd^{AB}*, *sd^{AC}*, *sd^{47M}*, or *IGMR>sd^{RNAi}*), like *yki* loss-of-function eyes, expressed Rh6 in almost all R8s with a corresponding loss of Rh5 (Fig. 1F and fig. S3E) and did not affect other photoreceptor subtypes (fig. S3, A to C). *sd* mutants or *sd^{RNAi}* also suppressed the *yki* gain-of-function phenotype (*sd*; *IGMR>yki*) (Fig. 1F and fig. S3, E and F), indicating that *sd* is required for *yki* function and likely encodes the Yki partner required for Rh5 and Rh6 regulation.

We next confirmed that *yki* acts canonically downstream of Wts to regulate Rhodopsin expression. In yR8s, Merlin (Mer) constitutively activates Hippo signaling to promote Wts activity (4). Their loss (*mer⁴* or *wts^{RNAi}*) led to Rh5 expansion, but *yki^{RNAi}* suppressed these phenotypes (Fig. 1G and fig. S4A); furthermore, ectopic *yki* (*GMR>yki*) strongly suppressed Hippo pathway-induced Rh6 (*GMR>wts*, *GMR>hpo*, or *GMR>wts>sav*) (fig. S4, B and C). We also tested whether *yki* and *melt* require each other to activate Rh5. Strong (*ey>IGMR>yki^{RNAi}*) or even mild (*yki^{B3/+}*) *yki* loss-of-function suppressed the ability of ectopic *melt* to induce Rh5 (fig. S4D). However, ectopic *yki* still induced Rh5 when *melt* was absent, even when expressed late in yR8s (*Rh6>yki*; *melt*) (Fig. 1H), consistent with *yki* functioning downstream of Melt and the Hippo pathway to regulate Rhodopsins. This regulation occurred through Wts-dependent Yki phosphorylation and inactivation because misexpression of dominant-negative kinase-dead (KD) forms of Wts or Hpo

¹Center for Developmental Genetics, Department of Biology, New York University, New York, NY 10003, USA. ²Division of Pediatric Ophthalmology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA. ³Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA.

*These authors contributed equally to this work.

†Present address: Department of Biology, Stanford University, Stanford, CA 94305, USA.

‡Present address: Department of Integrative Biosciences, Oregon Health & Science University, Portland, OR 97239, USA.

§Corresponding author. E-mail: tiffany.cook@cchmc.org (T.C.); claude.desplan@nyu.edu (C.D.)

in the retina (*IGMR>hpo^{KD}* or *IGMR>wts^{KD}*) (3) or constitutive gain-of-function alleles that reduce phosphorylation by Wts (*yki^{Dbo1}*, *yki^{Dbo2}*) (20) resulted in Rh5 expression in >70% of R8s (fig. S5) (3). Thus, the molecular relationship among Hippo pathway members in growth also exists in R8 fate.

Yki Creates Network-Level Hippo Pathway Positive Feedback by Regulating *wts* and *melt*

We next assessed whether *yki*-dependent feedback exists with its upstream regulators in R8s by removing retinal *yki* and *sd* function and assaying expression of *wts* (yR8s/Rh6) and *melt* (pR8s/Rh5). *yki^{RNAi}* or *sd^{47M}* mutant clones caused *wts-lacZ* expansion and *melt-lacZ* loss in most R8s (Fig. 2, A, B, and D), mirroring the gain of Rh6 and loss of Rh5. Conversely, activated *yki*

(*yki^{Dbo1/+}*, *IGMR>yki*, or *IGMR>yki^{S168A}:GFP*) expanded *melt-lacZ* into >85% of R8s, with correspondingly decreased *wts-lacZ* (Fig. 2C and fig. S6, A to C), and this function required *sd* (fig. S6, A to C). Therefore, in postmitotic photoreceptors, Yki promotes its own activity with positive network-level feedback by activating *melt* and repressing its direct negative regulator, *wts*—a regulation opposite from that in growth control.

Yki/Sd could regulate the *wts-melt* cross-repression by activating *melt*, repressing *wts*, or both. To determine the feedback mechanism, we first performed epistasis analysis between *yki* and *wts* while monitoring *melt* expression. In *wts^{RNAi}* retinas, *melt* expanded into all R8s, yet when *yki* was simultaneously removed (*IGMR>wts^{RNAi}+yki^{RNAi}*), *melt* expression was lost (Fig. 2D). Conversely,

ectopic *wts* failed to repress *melt* in the presence of ectopic *yki* (fig. S6C). Thus, *yki* does not activate *melt* by repressing *wts*; rather, it acts downstream of *wts* to promote *melt*.

If Yki repressed *wts* exclusively by inducing *melt*, *wts* should be derepressed in *melt* mutants even if upstream Hippo signaling is inactive. However, *wts-lacZ* was absent in *mer;melt* double-mutant R8s (fig. S6D), suggesting that a *melt*-independent factor(s) represses *wts* in *mer* mutants. We hypothesized that the factor(s) includes Yki. Indeed, *melt* failed to repress *wts* in the absence of *yki* or *sd* (Fig. 2D), and ectopic *yki* largely retained the ability to repress *wts* expression in the absence of *melt* (*melt; GMR>yki*) (Fig. 2E). Thus, Yki not only functions downstream of *wts* to activate *melt*, but also functions downstream of *melt* to repress *wts*.

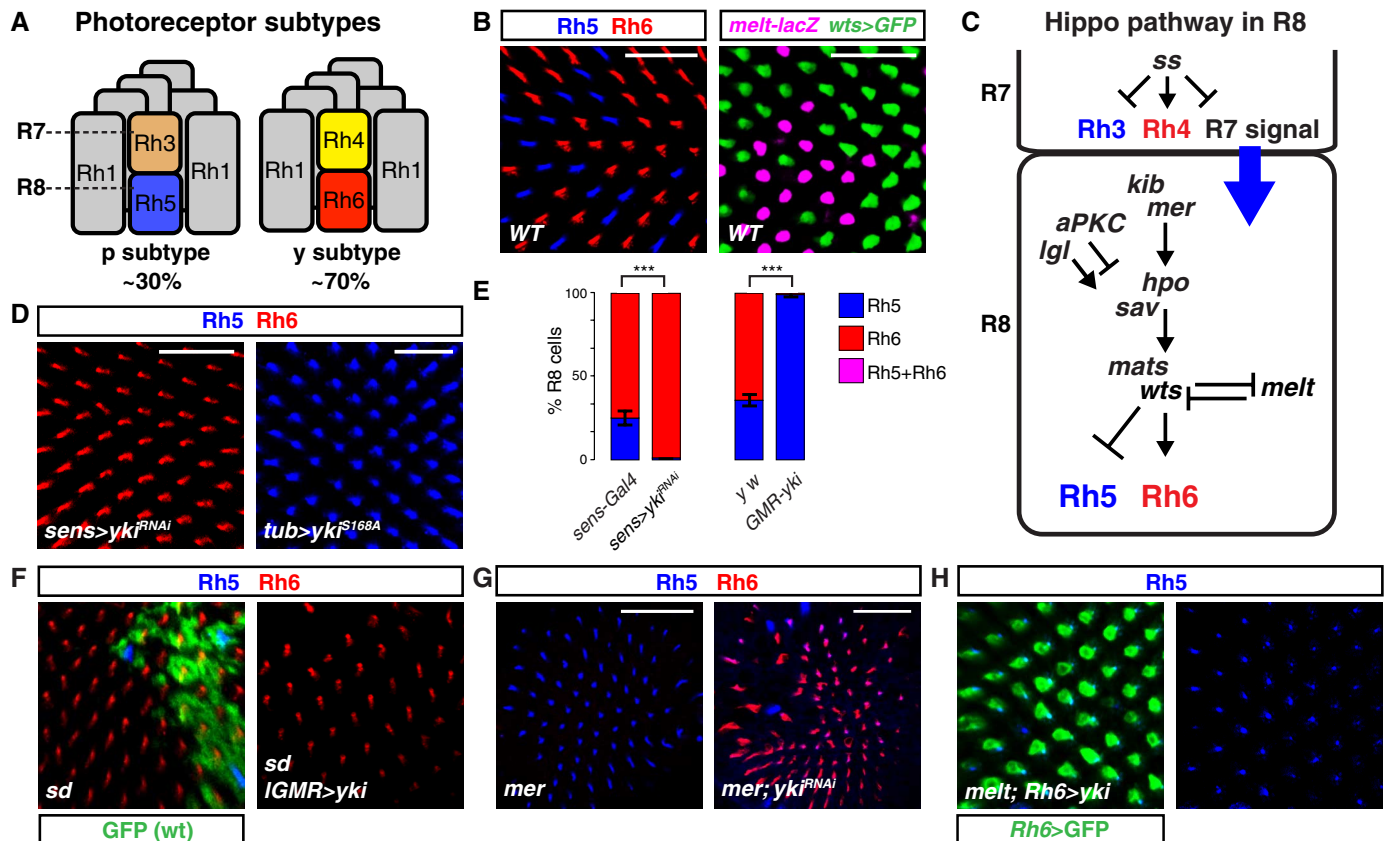


Fig. 1. Yki and Sd instruct mutually exclusive R8 neuron subtypes.

(A) Main photoreceptor subtypes and Rhodopsin coupling in the *Drosophila* eye. (B) Confocal images of whole-mounted, wild-type adult retinas. (Left) R8 subtypes visualized with antibodies to Rh5 (blue) and Rh6 (red); (right) R8 subtypes labeled with transcriptional reporters for *melt* [antibody against β -galactosidase (β -Gal), magenta] and *wts* (antibody against GFP, green). Scale bar, 25 μ m. (C) Hippo pathway regulation of R8 subtypes. R7 signals to R8 to induce pR8s (*melt* and Rh5). yR8 cells express *wts* and Rh6. *wts* and *melt* act in a double negative transcriptional feedback loop. Additional Hippo pathway members required to specify yR8 fate include the entire “core complex” (Wts, the Hpo kinase, the adapter protein Salvador (Sav), and Wts cofactor Mats) and the upstream regulators Lethal Giant Larvae (Lgl), the FERM-domain protein and NF-2 ortholog Merlin (Mer), and the WW-domain protein Kibra (Kib) (4). Atypical protein kinase C (aPKC) antagonizes yR8 fate. Black arrows and lines represent genetic regulatory interactions. (D) *yki* is necessary and sufficient to specify pR8 fate. *yki* knockdown (*yki^{RNAi}*) (left). Pan-photoreceptor expression of activated Yki (right), induced by *GMR-flp/FRT*-mediated excision of a

transcriptional STOP between *tubulin* promoter and *yki^{S168A}* (*GMR-flp, tub-FRT-STOP-FRT- yki^{S168A}*). Scale bar, 25 μ m. (E) Effect of *yki* manipulations on percentage of R8 cells expressing Rh5 or Rh6 (y axis). Yki is necessary (left) and sufficient (right) to induce Rh5. Wild-type range is ~20 to 40% Rh5. From left to right in graph: *sens-Gal4* ($n = 10$, $N = 2998$ R8 neurons), *sens-yki^{RNAi}* ($n = 8$, $N = 2112$), *y, w* ($n = 14$ retinas, $N = 2790$), *GMR-yki* ($n = 4$, $N = 510$). Error bars are $\pm$ SD; two-tailed, unpaired Student's *t* test; $^{**}P < 0.01$, $^{***}P < 0.001$. (F) *sd* is required for pR8 fate. (Left) *sd^{47M}* mutant clone (GFP absence). The total number of ommatidia was not reduced, indicating that R8 cells were misspecified into yR8 rather than pR8 cells being lost. (Right) Yki misexpression in *sd* mutant background. (G) Yki acts downstream of the Hippo pathway and *melt* to control Rh5 and Rh6. (Left) *mer⁴*; (right) *IGMR>yki^{RNAi}* suppresses the *mer⁴* phenotype. Scale bar, 50 μ m. (H) Ectopic expression of *yki* in the opposite subtype with *Rh6-Gal4* induces Rh5 in *melt^{Δ1}* mutants. (Left) *Rh6-Gal4*-expressing cells colabeled by expression of GFP (green). (Right) Rh5 channel only. Except where noted, in all manuscript figures, Rh5 and Rh6 are labeled in blue (Rh5) and red (Rh6).

If ectopic Yki can repress *wts* independently of *melt*, what is the role of *melt*? Although all R8s expressed Rh6 in *melt* mutants, up to 20% of R8s still coexpressed Rh5 in 1-day-old adults, which decreased to < 8% after 3 weeks (fig. S7). Because

no such delay was observed when *yki* was removed, and because *yki* activation consistently induces Rh5 in 100% of R8s, we infer that the transient Rh5 expression in young *melt* mutant flies reflects transient Yki activity. The above data suggest that Melt

and Yki are temporally separated: Melt is not required to initiate pR8 fate in response to the pR7 signal, but instead likely consolidates Wts inactivity and Yki activity immediately after the fate decision to ensure robust Yki function in pR8s.

Fig. 2. Yki and Sd regulate *wts* and *melt* expression in Hippo pathway positive feedback.

(A) *yki^{RNAi}* retinas (top right) contain *wts-lacZ* (green) in almost all R8s, compared to about two-thirds of R8s in wild-type controls (top left); *melt-lacZ* (blue) is absent from most R8s in *yki^{RNAi}* retinas (bottom right) compared with controls (bottom left). Top panels: antibodies to β -Gal (*wts-lacZ*) and Rh6 (red). Bottom panels: β -Gal (*melt-lacZ*), Rh5 (green), and Rh6 (red). Images at R8 nuclei focal plane. Scale bar, 10 μ m.

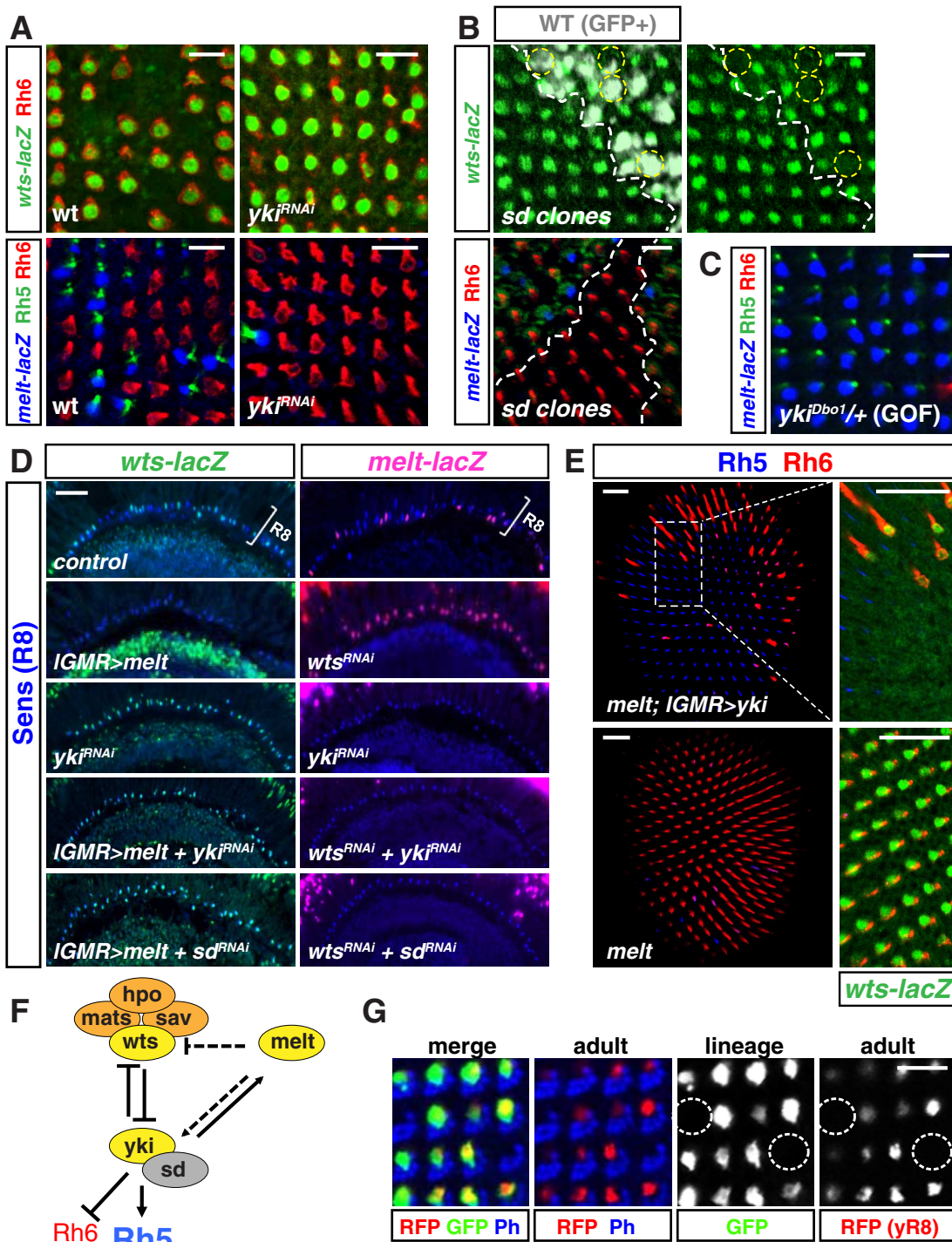
(B) *sd* mutant clones (absence of GFP) labeled with antibodies to β -Gal to mark *wts-lacZ* (top, green) and *melt-lacZ* (bottom, blue) transcriptional reporters. Dashed circles in top panels show wild-type R8s without *wts-lacZ*, whereas all *sd* mutant R8s contain *wts-lacZ*. Dashed lines indicate clone boundary. Bottom panel also stained for Rh6. Scale bar, 10 μ m.

(C) A heterozygous *yki* gain-of-function (GOF) allele is sufficient to induce *melt* expression in most R8s. Images in focal plane of R8 nuclei. Antibodies to β -Gal (blue), Rh5 (green), and Rh6 (red). Scale bar, 10 μ m.

(D) Sagittal sections of adult eyes with nuclei stained for the R8 marker Sens (blue) and *wts* (β -Gal; green) or *melt* (β -Gal; magenta) expression. (Left) *melt* requires *yki* and *sd* to repress *wts*. *wts-lacZ* is absent from R8s in *IGMR>melt* retinas, but derepressed when *yki* or *sd* are simultaneously removed (bottom two panels). (Right) *melt-lacZ* is expressed in most R8s in *IGMR>wts^{RNAi}* retinas, but is lost when *yki* or *sd* are removed. White bracket denotes R8 layer. Scale bar, 50 μ m.

(E) Top: *melt; IGMR>yki:GFP* adult retina labeled for Rh5 and Rh6 (left). Yki can repress *wts* (β -Gal, green in right) in most R8s in the absence of *melt*. (Right) Panels shows R8 nuclear layer. A minority of R8s still express *wts*, but most do not. Compare to *melt* mutant retina, where *wts* is expressed in almost all R8s (bottom right). Scale bar, 25 μ m.

(F) Model of Wts-Yki-Melt feedback circuit in R8 subtypes. Regulatory arrows are genetic and show transcriptional control, except for Wts inhibition of Yki, which is biochemical and posttranscriptional. Dashed lines indicate non-mutually



exclusive interactions. **(G)** Lineage-tracing experiment for *wts-Gal4* expression in R8 subtypes using G-TRACE (45). Adult retinas stained for RFP (red), GFP (green), and phalloidin (Ph; blue). RFP alone labels contemporary, adult *wts-Gal4* expression. GFP labels *wts-Gal4* expression lineage. Right two panels are grayscale of RFP and GFP, respectively. Dashed circles show that pR8 cells did not express *wts-Gal4* in their history. Scale bar, 10 μ m.

Together, these data indicate that Yki regulates both *wt*s and *melt* to promote pR8 specification and reveal that Yki network-level positive feedback occurs through two mechanisms (Fig. 2F): (i) a Wts-Yki double negative-feedback loop, wherein Wts inactivates Yki biochemically and Yki represses *wt*s transcription; and (ii) a double positive-feedback loop between Melt and Yki, wherein Yki activates *melt* expression, and Melt promotes Yki (or inhibits the Hippo pathway to activate Yki). This combination ensures a complete switch from the default (yR8) to the induced (pR8) fate.

The R8 Hippo Network Is Distinct from the Hippo Growth Regulation Network

To evaluate the context specificity of the R8 Hippo network, we asked whether *wt*s transcriptional regulation is specific to postmitotic R8 cells. We used the G-TRACE lineage reporter (27) to simultaneously label historical [green fluorescence protein (GFP)] and contemporary [red fluorescence protein (RFP)] *wt*s-*Gal4* expression and found that GFP was only coexpressed with RFP in yR8s (Fig. 2G). This indicates that *wt*s-*Gal4* was not expressed earlier in mitotic pR8 progenitor cells and instead is activated postmitotically to control R8 subtype specification.

We also asked whether the previously unknown Hippo regulatory relationships in R8—*melt* repressing *wt*s, *yki*/*sd* repressing *wt*s and activating *melt*—also exist in growth contexts. First, we examined *wt*s-*lacZ* expression after Hippo pathway manipulation in the posterior compartment of the larval wing disc using an *engrailed* (*en*)–*Gal4* driver. Unlike in R8s, neither *yki*^{RNAi} nor *wt*s misexpression increased *wt*s-*lacZ* expression. Furthermore, in third-instar larval eye discs, we did

not detect up-regulation of *wt*s-*lacZ* in *sd* mutant clones (fig. S8A) or of *melt*-*lacZ* in *wt*s mutant clones (fig. S8B). This suggests that the Hippo pathway does not regulate the expression of *wt*s or *melt* in dividing epithelial cells of the wing or eye disc.

We next tested whether the Wts-Yki-Melt regulatory circuit exists in growth control by manipulating *melt* and assaying the Yki growth target Ex. Whereas ectopic *yki* or *wt*s^{RNAi} (which phenocopy *melt* gain-of-function in R8) autonomously induced Ex levels when expressed in the wing, ectopic *melt* did not increase Ex protein or *ex* transcription (*ex*-*lacZ*) (fig. S8D). Although *en*>*melt* adult wings were slightly larger than *en*-*Gal4* control wings, strong overexpression of *melt* in the developing and adult eye did not noticeably affect eye size or morphology (fig. S8C). This difference is likely due to *melt*'s known role in the wing as a growth promoter in the insulin/target of rapamycin (TOR) pathway (22), which is dispensable for *melt*-dependent R8 subtype determination (3). Collectively, these experiments indicate that the regulatory architecture and transcriptional feedback mechanism of the Hippo network differ between R8 subtype specification and growth regulation in at least two mitotically active tissues.

A Conserved Feedforward Otd-Tj (Crx-Nrl) Module Regulates Photoreceptor Subtypes

Given the differences in Yki-mediated feedback in growth and R8 fate, we investigated how the Wts-Yki-Melt regulatory circuit is established specifically in R8s but not in growth. Because *melt* is a context-specific inhibitor of the Hippo pathway, we focused on mechanisms underlying *melt* regulation in the eye. We generated serial

deletions of the *melt* first intron (4 kb), previously shown to confer expression in pR8s (3), and identified a 450–base pair (bp) (*melt*450-*lacZ*) element that drives reporter expression in pR8s (and pR7s) and responds appropriately to *wt*s in R8 (Fig. 3, A and B, and fig. S9, A and B). *melt*450 contains two conserved K₅₀ homeodomain (HD) binding sites whose mutations abolished reporter expression (Fig. 3, A and B, and fig. S9A), suggesting that a K₅₀ factor directly promotes *melt* expression. A good candidate was the pan-photoreceptor K₅₀ HD transcription factor Otd (23), which directly activates *Rh5* in pR8s and controls proper pR8:yR8 ratios (24–26) (fig. S9C). Consistent with *otd* being required for *melt* transcription, *melt*-*lacZ* was lost from all R8s when *otd* was removed (*sens*>*otd*^{RNAi}) (Fig. 3B), and *melt*450-*lacZ* was lost in eye-specific *otd*^{uni} mutants (fig. S9B). Moreover, Otd was sufficient to activate a *melt*450-*luciferase* (*luc*) reporter 3-fold in cultured *Drosophila* S2 cells, requiring intact K₅₀ sites (Fig. 3C). Thus, Otd directly activates expression of both the fate determinant *melt* and its downstream output *Rh5*, generating feedforward regulation to promote pR8 fate (Fig. 3C).

Because Otd is expressed in all photoreceptors, we posited that other factors may act with Otd to regulate the Hippo pathway in R8. We performed a photoreceptor-specific RNAi screen and identified *traffic jam* (*tj*), which encodes a basic leucine zipper (bZIP) transcription factor (27). *tj* knockdown in all photoreceptors, or a null *tj* allele that we generated (*tj*^{Δ1}), each led to loss of Tj protein and a significantly reduced Rh5:Rh6 ratio (Fig. 4, A to C, and fig. S10, B and C) without affecting R7 opsins (fig. S10D), suggesting that Tj is required for pR8 fate.

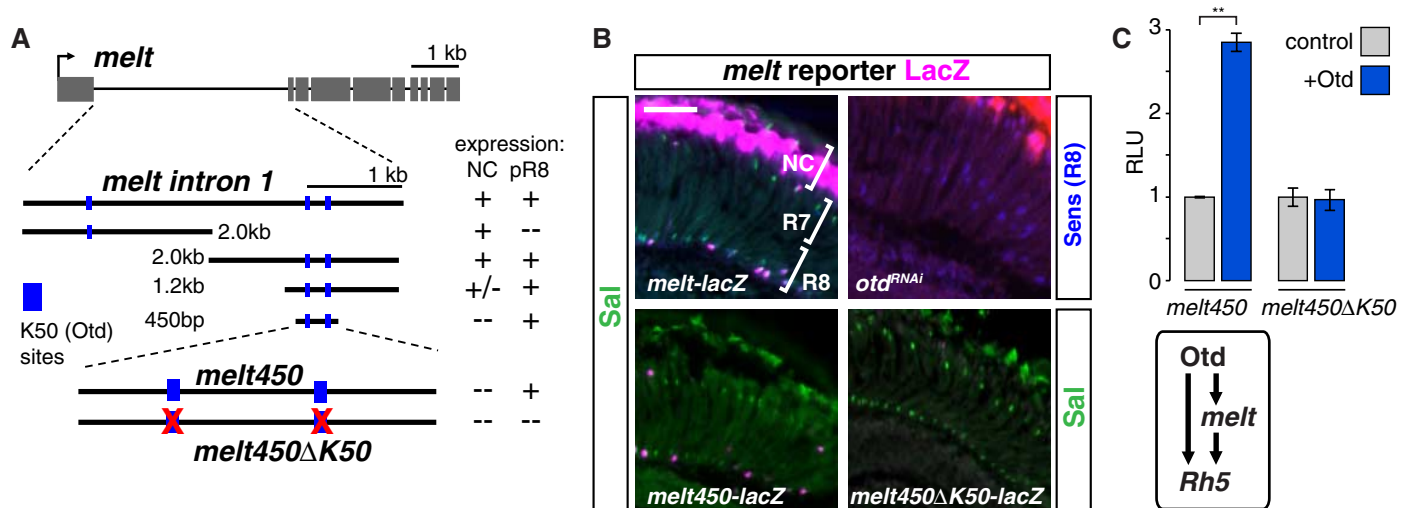


Fig. 3. The photoreceptor regulator Otd promotes *melt* expression in pR8s. (A) Schematic diagram of *melt* locus and genomic DNA fragments tested for pR8 expression. Blue boxes: K₅₀/Otd binding sites (TAATCC). Red Xs: Otd site mutations. (B) (Top left) A 4-kb intronic *melt*-*lacZ* reporter expresses in a subset of Sal-positive R8 cells and distal nonneuronal cells ("NC"). (Bottom left) A 450-bp *melt* enhancer is expressed in pR8s (and some pR7s). (Top right) Adult *otd* loss-of-function retinas immunostained for β-Gal (magenta) and the R7/R8 marker Sal (green). (Bottom right)

Mutation of two K₅₀/Otd binding sites in the *melt*450 enhancer (*melt*450ΔK50-*lacZ*) abolishes reporter expression. Scale bar, 50 μm. (C) Luciferase reporter assays in S2 cells. y axis: relative luciferase units (RLU) normalized to a control that represents cells transfected with empty expression vector (= 1 RLU). Otd activates the *melt*450 enhancer about threefold, but does not activate *melt*450ΔK50. Error bars are ±SD; *n* = 3; ***P* ≤ 0.01. Bottom box: Otd and Melt form a feedforward loop to promote Rh5 expression (pR8 fate).

Tj retinal expression preceded Rhodopsin expression and was restricted to all R7s and R8s from ~60 hours after pupal formation (APF) into adulthood (Fig. 4F and fig. S10A). *tj* knockdown in R8 (*sens>tj^{RNAi}*) phenocopied *tj^{Δ1}* mutants, whereas R7 knockdowns (*sev>tj^{RNAi}*) had wild-type Rh5:Rh6 ratios (fig. S10, B, C, and E), indicating that *tj* autonomously affects R8 fate.

Consistent with Tj's role in promoting pR8 fate, *melt-lacZ* was lost and *wts-lacZ* was expanded into most *tj* mutant R8s (Fig. 4D and fig. S11A). Epistasis experiments revealed that *tj* both promotes *melt*, independently from *wts* (*wts^{p1}; IGMR>tj^{RNAi}; melt-lacZ*) (Fig. 4D and fig. S11A), and is necessary for *melt* to fully repress *wts* (*tj^{Δ1}; IGMR>melt; wts-lacZ*) (Fig. 4D and fig. S11A). Furthermore,

although Rh5 was uniquely expressed in all R8s in *melt* gain-of-function or *wts* loss-of-function retinas, simultaneous removal of *tj* in either situation resulted in Rh5:Rh6 coexpression in most R8s (Fig. 4E and fig. S11B). This indicates that *tj* functions downstream of *wts* and *melt* to repress Rh6, but does not activate Rh5. Combined, these experiments reveal that Tj controls pR8 fate through

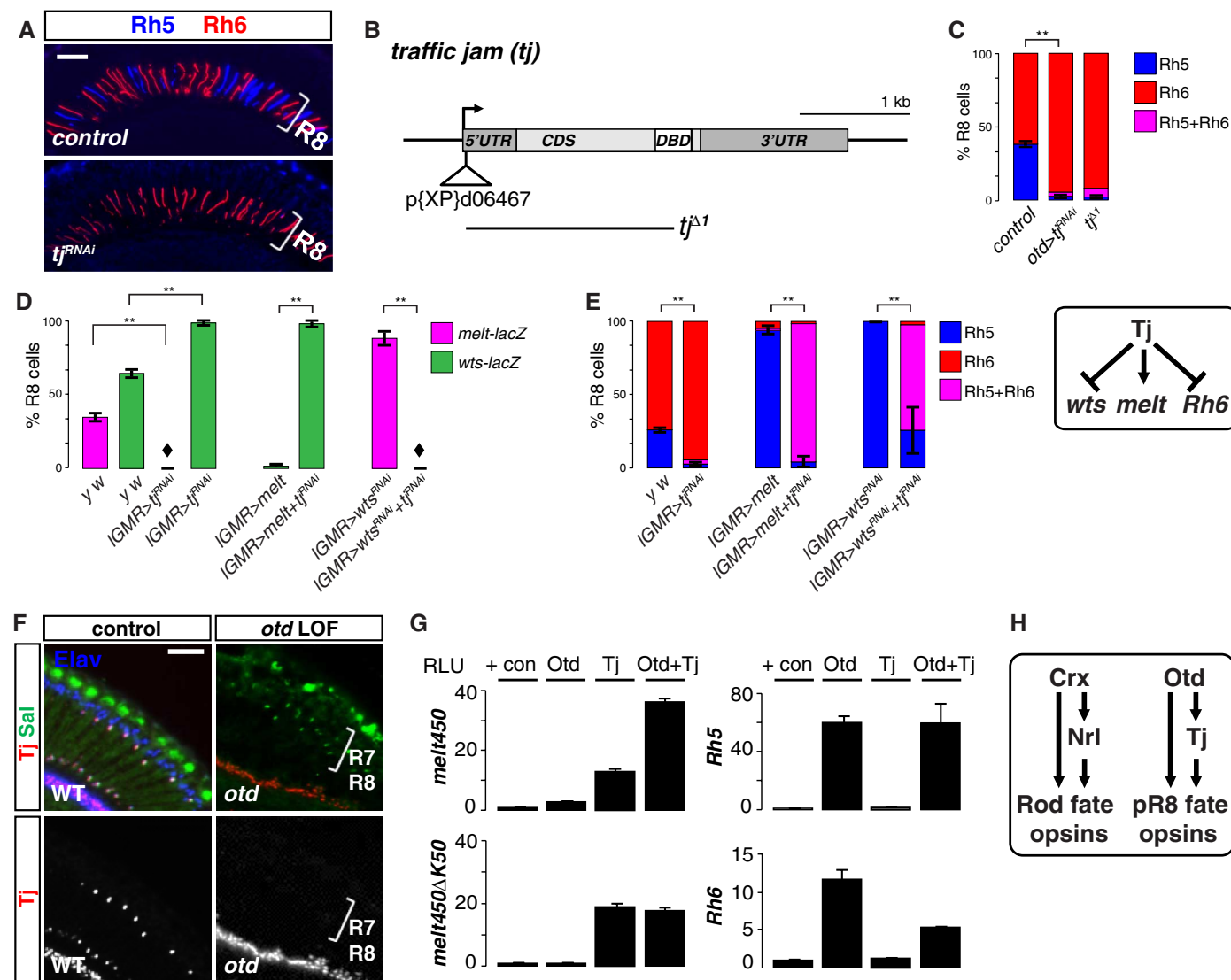


Fig. 4. Tissue-restricted transcription factors control *melt* and Rhodopsins with regulatory logic conserved in mammalian eye development. (A) Retinas expressing *tj^{RNAi}* lose Rh5 and gain Rh6. Scale bar, 50 μ m. (B) Diagram of the *tj* locus. *tj^{Δ1}* was generated by imprecise excision of a P element (triangle) in the 5' untranslated region (UTR). The *tj^{Δ1}* null mutant deletion is indicated by the black line. (C) Quantification of Rh5 and Rh6-expressing R8s. From left to right in graph: control (*otd-GAL4*): (*n* = 5, *N* = 997), *otd>tj^{RNAi}* (*n* = 4, *N* = 898), *tj^{Δ1}* (*n* = 6 retinas, *N* = 1230 R8 neurons). Error bars are SD; ***P* < 0.01. (D) Tj regulates *wts* and *melt*: Quantification of *wts*- and *melt*-expressing R8s. From left to right: control (*yw*): (*n* = 4, *N* = 920), *IGMR>tj^{RNAi}* (*n* = 7, *N* = 1411), *IGMR>melt* (*n* = 4, *N* = 980), *IGMR>melt+tj^{RNAi}* (*n* = 4, *N* = 892), *IGMR>wts* (*n* = 5, *N* = 912), *IGMR>tj^{RNAi} + wts^{RNAi}* (*n* = 4, *N* = 760); error bars are SD; ***P* < 0.01. Diamonds denote complete loss of *melt-lacZ*. (E) Tj regulates Rh6 downstream of *wts* and *melt*. From left to right: *y w* control (*n* = 6, *N* = 1185), *IGMR>tj^{RNAi}* (*n* = 8, *N* = 1675), *IGMR>melt* (*n* = 5, *N* = 1081), *IGMR>melt+tj^{RNAi}* (*n* = 5, *N* = 1021), *IGMR>wts* (*n* = 4, *N* =

782), *IGMR>tj^{RNAi} + wts^{RNAi}* (*n* = 4, *N* = 774). Percent Rh5 compared; error bars are SD. ***P* < 0.01. Right diagram: Tj regulates *wts*, *melt*, and Rh6. (F) Tj is expressed in R7 and R8, but not in *otd* mutants. Adult sections; (top left) wild-type retinas labeled for the neuronal marker Elav (blue), the R7/R8 marker Sal (green), and Tj (red). (Bottom left): Tj alone in gray-scale in wild-type retinas. (Right) *otd* loss-of-function (LOF) mutants (*otd^{uvr}*) lose Tj (red); Sal (green). Scale bar, 50 μ m. (G) Luciferase reporter assays in S2 cells. (Top left) Tj activates *melt450* expression ~12-fold, whereas Otd and Tj synergistically activate *melt450* ~37-fold; (bottom left) mutating *K50*/Otd sites in *melt450* abolishes Otd-dependent, but not Tj-dependent, activation. (Top right) Otd, but not Tj, activates *Rh5* promoter expression. (Bottom right) Tj can repress Rh6. Error bars are $\pm$ SD; *n* = 3. (H) A OTX/CRX/Otd-MAF/NRL/Tj coherent feedforward motif instructs photoreceptor fate in flies and mammals. Crx (Otd) promotes expression of Nrl (Tj), and both factors promote a specific photoreceptor fate and Rhodopsin expression. Left, mammalian motif; right, fly motif.

three distinct mechanisms: Tj promotes *melt*, represses *wts*, and represses *Rh6* (Fig. 4E, right).

Tj is the single *Drosophila* ortholog of the four mammalian MAF-bZIP transcription factors (28). One MAF factor—Nrl (neural retina leucine zipper)—is a target of the Otd orthologs Otx2 and Crx and functions synergistically with Crx in the mouse retina to promote rod photoreceptor formation at the expense of cones (29–31). Given that *otd* and *tj* also promote one photoreceptor fate (pR8s) at the expense of another (yR8s), we examined the genetic relationship between *otd* and *tj* in the fly retina. Otd was unaffected in *tj*^{RNAi} retinas (fig. S11C), but Tj was absent in *otd*^{RNAi} mutants (Fig. 4F). Thus, Otd promotes *tj* expression in fly photoreceptors, analogous to how Otx2/Crx promotes *Nrl* expression in mammalian rods. Otd-dependent pR8 gene expression, however, is not just a consequence of activating *tj*, as resupplying *tj* in *otd*^{RNAi} mutants did not restore *melt* (fig. S11, D and E). Hence, *otd* acts upstream of *tj*, but both are required to promote *melt* expression. Thus, similar to Crx and Nrl regulation of mammalian photoreceptor fate, Otd and Tj form a coherent feedforward loop that promotes pR8 fate (Fig. 4H).

This model predicts that Otd and Tj cooperate to promote *melt*. Indeed, although no MAF consensus DNA binding sites were detected in the *melt450* enhancer, Tj was sufficient to induce *melt450-luc* 12-fold and synergistically increased Otd-dependent activation from ~3-fold to 35-fold in S2 cells (Fig. 4G). Similar to our in vivo results, Tj did not induce *Rh5* expression in S2 cells, but did repress *Rh6* promoter activity (Fig. 4G). Combined, our in vivo and in vitro results uncover a

second and conserved feedforward system in pR8s, wherein Otd induces *tj* and Otd and Tj together activate *melt* expression in pR8s.

Yki Requires the Conserved Otd-Tj Module to Induce pR8 Fate

Because Otd and Tj are expressed in all R8s, whereas Yki/Sd function is biochemically restricted to pR8s by Wts, we asked whether these transcriptional regulators integrate to promote pR8 fate. Consistent with *otd* and *tj* being essential for *melt* activation, *yki* misexpression failed to induce *melt* in *otd*^{RNAi} or *tj*^{RNAi} eyes (Fig. 5A). Moreover, *yki* failed to activate *Rh5* in *otd*^{RNAi} flies or repress *Rh6* in *tj*^{RNAi} eyes (fig. S12A). Thus, *yki* requires *otd* and *tj* activity to exert its pR8 specification functions: (i) *yki* requires *otd* and *tj* to induce *melt*, (ii) *yki* requires *otd* to activate *Rh5*, and (iii) *yki* requires *tj* to repress *Rh6* (Fig. 5B, right).

To test this integration molecularly, we analyzed the ability of Yki+Sd to influence Otd- and Tj-dependent activation of *melt* and *Rh5* in S2 cells. Yki+Sd weakly activated *melt* (~3-fold), additively increased Tj-dependent activation (from 8- to 12-fold), and synergistically increased Otd-dependent activation of *melt* (from ~3- to 20-fold) (Fig. 5B). However, the highest *melt* activation was observed with Otd, Tj, Sd, and Yki together (60-fold), consistent with the requirement of all four factors for pR8 fate in vivo. Similarly, Yki and Sd minimally activated the *Rh5* promoter (~3-fold), but largely increased Otd-dependent activation (from 60- to 125-fold) (Fig. 5B). Although the K₅₀/Otd sites were necessary for expression of *melt* and *Rh5*, mutating potential Sd sites in the

Rh5 promoter did not decrease reporter expression in vivo, suggesting that Yki/Sd-dependent activation of *Rh5* occurs indirectly. These studies support the model that Otd, Tj, and Yki/Sd cooperate to promote pR8-specific gene expression.

Together, our results indicate that the pR8 state depends on two overlapping feedforward regulatory networks: (i) Otd directly promotes *melt* and *Rh5* expression (Fig. 3, A to C) (24, 25). *Melt* then further promotes *Rh5* by antagonizing the Hippo pathway and promoting Yki activity; (ii) Otd promotes *tj* expression, and Tj and Otd then synergistically induce *melt*, while Tj also represses *wts* and *Rh6*. Because Yki requires Otd and Tj to induce *melt* (which ultimately promotes Yki), Otd/Tj provide a critical transcriptional context for Yki positive feedback in R8.

Photoreceptor- and R8-Restricted Transcription Factors Promote yR8 Fate

We next investigated the mechanisms controlling the yR8 “default” state (active Hippo pathway and *Rh6*). *Sens*, an R8-restricted zinc finger transcription factor necessary early for R8 specification (32) and later for terminal R8 differentiation (25, 32, 33), and Pph13, a pan-photoreceptor Q₅₀ homeodomain transcription factor, are both essential for *Rh6* expression (34). Thus, we tested whether these factors also promote yR8 fate. Removing *sens* late (*sens>sens*^{RNAi}) reduced *wts* expression (fig. S13A), indicating that *Sens* functions in yR8s to promote *wts* and *Rh6* expression (Fig. 5C). *Sens* did not, however, strongly affect pR8 fate as *Rh5* was only mildly affected (Fig. 5C). In *pph13*^{hazy}-null mutant retinas, not only were *wts*

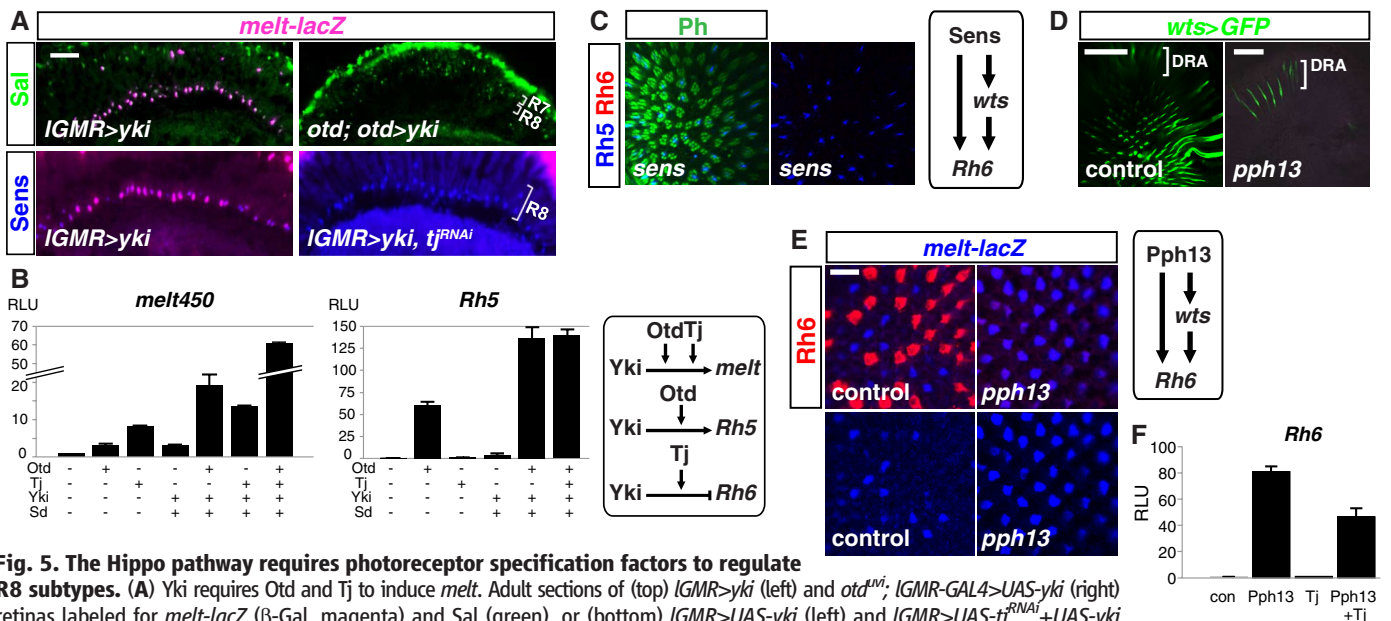


Fig. 5. The Hippo pathway requires photoreceptor specification factors to regulate

R8 subtypes. (A) Yki requires Otd and Tj to induce *melt*. Adult sections of (top) *IGMR>yki* (left) and *otd*^{RNAi}; *IGMR-GAL4>UAS-yki* (right) retinas labeled for *melt-lacZ* (β-Gal, magenta) and Sal (green), or (bottom) *IGMR>UAS-yki* (left) and *IGMR>UAS-tj*^{RNAi} + *UAS-yki* (right) labeled for *melt-lacZ* (magenta) and Sens (blue). Scale bar, 50 μm. **(B)** Yki, Sd, Otd, and Tj synergistically activate *melt*

and *Rh5*. Luciferase reporter assays for *melt* and *Rh5* enhancer activity in S2 cells. Cells were transfected with indicated combinations of Otd, Tj, Yki, and Sd. **(Right)** Yki requires Otd and Tj to activate *melt* and Yki requires Otd to activate *Rh5*. Error bars are ±SD; *n* = 3. **(C)** *sens* late-mutant retinas (45) lose *Rh6* and *wts* expression; some *Rh5* remains. **(D)** *pph13* mutant retinas lose *wts* expression. *wts>GFP* remains expressed in R8s of the dorsal rim area (DRA) R8s (bracket) at the margin of the retina, but is absent in yR8s. Scale bars, 50 μm. **(E)** (Left) *pph13* mutants gain *melt-lacZ* (blue) in most R8s. (Right) Pph13 is required for *wts* and *Rh6* expression and to specify yR8 fate. Scale bar, 10 μm. **(F)** Tj reduces Pph13-mediated activation of *Rh6* promoter. Error bars are ±SD; *n* = 3.

and *Rh6* expression lost, but *melt* was also expanded into most R8s (Fig. 5, D and E). In addition, the *Rh6* promoter required the Pph13/Q₅₀ HD binding site for its *in vivo* activity (fig. S13C), supporting the notion that Pph13 binds to and activates the *Rh6* promoter (34). Rh5 protein was difficult to assess owing to *ppl13*'s role in forming rhabdomeres, where Rh5 protein localizes. Nevertheless, *Rh5-GFP* remained expressed (fig. S13B), confirming that Otd, but not Pph13, is required for *Rh5* promoter activity (34). Therefore, *ppl13* regulates yR8 fate determinants (it promotes *wt*s and represses *melt*) and directly activates *Rh6* (Fig. 5E), in another feedforward loop resembling Otd-Tj-Melt and Otd-Melt-Rh5 regulation in the alternate subtype.

Since Sens and Pph13 are expressed in all R8s, what prevents these factors from activating yR8 gene expression in pR8s? Tj plays this role for Rh6, as Pph13 strongly activates *Rh6* promoter activity (~80-fold) and Tj represses this activation (Fig. 5F). Because Rh5 persists in *sens* and *ppl13* mutants, these factors are likely to be permissive to promote *wt*s and *Rh6* in all R8s.

Together, these results indicate that the R8 Hippo network topology requires Otd, Tj, Pph13, and Sens activity to permissively promote both subtypes in all R8s. Such regulation endows any R8 with competence to respond to the stochastically expressed signal from pR7. Ultimately, the Yki-Wts-Melt feedback module provides the instructive switch that decides between default (yR8) and acquired (pR8) states (Fig. 6A).

Discussion

A fundamental strategy in animal development is to repurpose the same signaling pathways for diverse functions. We identified a tissue-specific transcription factor network that enables the otherwise homeostatic Hippo growth control pathway to act as a bistable switch for terminal cell fate. This alteration in network-level properties—such as positive versus negative feedback—within biochemically conserved pathways is an efficient means to reuse a signaling network in contexts as distinct as proliferation and terminal differentiation.

How is the R8-specific Hippo regulatory circuit achieved? The two interlinked Yki positive feedback loops (one with *wt*s, one with *melt*) provide the R8 Hippo pathway with multiple points of potential regulation. Context-specific expression of *wt*s and *melt* is defined by overlapping expression of four transcription factors: Otd, Tj, Pph13, and Sens (Fig. 6A). Otd and Pph13 are expressed in all photoreceptors and generate a permissive context that endows the initially equipotent R8s with the competence to become either subtype: Otd promotes *melt*/Rh5 whereas Pph13 promotes *wt*s/Rh6 expression. This competence is further restricted by expression of Tj in R7 and R8, and Sens in R8s, which ensures that *melt* and *wt*s cross-regulation is restricted to R8s. Importantly, the status of Yki activity and resulting feedback assures the outcome of p versus y fate: In pR8s, Yki functions with Otd and Tj to promote *melt* and *Rh5*; in yR8s, *wt*s inhibits Yki, pre-

venting *melt* and *Rh5* expression and allowing “default” *wt*s and *Rh6* expression by Pph13 and Sens. Each of these four transcription factors regulates a partially overlapping subset of R8 subtype fate genes, and together, the network cooperates at multiple regulatory nodes to provide the specific context for repurposing the Hippo pathway.

Although other instances of pathways with both positive and negative feedback exist, these are conceptually different from R8 Hippo regulation. For example, in Sprouty (hSpry) regulation of Ras/MAPK-mediated epidermal growth factor receptor (EGFR) signaling, EGFR induces hSpry2 expression but hSpry2 inhibits EGFR function (negative feedback); however, hSpry2 also promotes EGFR activity by preventing Cbl-dependent EGFR inhibition (positive feedback) (35, 36). hSpry2 positive feedback is likely coupled to its

negative feedback to fine-tune the length and amplitude of receptor activation (36). By contrast, the opposite Hippo pathway feedbacks occur in vastly different cell types (mitotic epithelial cells versus postmitotic neurons), and both forms of feedback are unlikely to coexist in R8 because Yki's repression of *wt*s expression (positive feedback) would render Yki up-regulation of upstream Wts activators (negative feedback) inconsequential.

Gaining positive feedback or losing negative feedback within Hippo signaling could permit oncogenesis. Indeed, the *yki* ortholog, *YAP*, is an oncogene (37, 38) and is amplified in multiple tumors, and *LATS1/2* (*wt*s) down-regulation is associated with non-small cell lung carcinomas, soft tissue sarcoma, metastatic prostate cancers, retinoblastoma, and acute lymphoblastic leukemia (39). Otx and MAF factors are also oncogenic in a num-

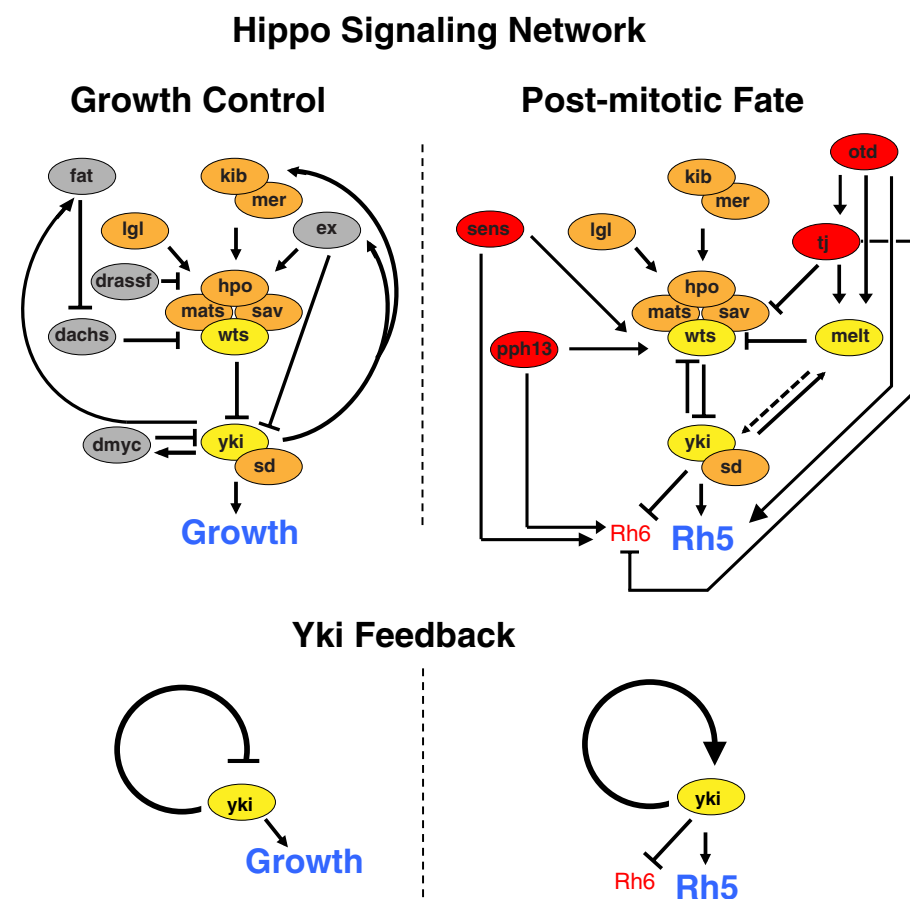


Fig. 6. Tissue-restricted transcription factors promote reuse of the Hippo pathway with positive feedback for postmitotic neuronal fate. (A) A functionally conserved cassette of genes from Kib/Mer to Yki/Sd is rewired for a context-specific purpose through changes in network-level feedback. Comparison of network-level feedback switch in Hippo pathway for growth versus postmitotic R8 fate. In growth control (left), at least four negative feedbacks onto Yki generate homeostatic regulation for Yki's growth-promoting function. In postmitotic R8 fate specification, positive feedback onto Yki induces an all-or-nothing decision to become pR8 and express Rh5. Four tissue-specific transcription factors (red ovals), including the conserved Otd-Tj (OTX-NRL) module, are coexpressed only in R8 in the eye and generate the permissive context for a Wts-Yki-Melt regulatory circuit and Yki positive feedback. Gray ovals are Hippo growth pathway genes not involved in R8 subtype specification. Orange ovals are genes involved in both contexts. Yellow ovals are genes that create the R8 feedback mechanism and are yellow in the growth pathway for comparison. (B) Effect of Hippo pathway regulatory interactions on network-level feedback onto Yki, in growth control (left; negative feedback) or postmitotic neural differentiation (right, positive feedback).

ber of tissues (40, 41). Thus, understanding whether the regulatory interactions identified here function in other cellular contexts will be crucial for deciphering how normal Hippo signaling could go awry.

Our findings also reveal that a Crx/Otd-Nrl/Tj feedforward module plays a conserved role in postmitotic photoreceptor fate specification in both flies and mammals. Both induce one photoreceptor fate at the expense of another, and both regulate opsins with a feedforward loop wherein Crx/Otd activates Nrl/Tj expression and Crx-Nrl or Otd-Tj synergistically activate downstream targets (31). Given such deep evolutionary conservation, this module may be critical for generating photoreceptor diversity in other complex visual systems.

This study has two main implications. First, although positive feedback is well documented in other switch-like, irreversible cell fate decisions such as in *Xenopus* oocyte maturation or cell cycle entry (42–44), our work suggests that positive feedback could have a broad role in terminal neuronal differentiation, which often requires permanent fate decisions to maintain a neuron's functional identity. Second, the changes in network topology in R8 photoreceptors allows a finely tuned growth control pathway to be used as a switch in a permanent binary cell fate decision. Context-specific regulation allows the feedback architecture to change in an otherwise conserved signaling module. This may be a general mechanism to endow signaling networks with new systems properties and diversify cell fates in development and evolution.

References and Notes

1. A. Pires-daSilva, R. J. Sommer, The evolution of signalling pathways in animal development. *Nat. Rev. Genet.* **4**, 39–49 (2003). doi: [10.1038/nrg977](#); pmid: [12509752](#)
2. G. Halder, R. L. Johnson, Hippo signaling: Growth control and beyond. *Development* **138**, 9–22 (2011). doi: [10.1242/dev.045500](#); pmid: [21138973](#)
3. T. Mikeladze-Dvali *et al.*, The growth regulators warts/lats and melted interact in a bistable loop to specify opposite fates in *Drosophila* R8 photoreceptors. *Cell* **122**, 775–787 (2005). doi: [10.1016/j.cell.2005.07.026](#); pmid: [16143107](#)
4. D. Jukam, C. Desplan, Binary regulation of Hippo pathway by Merlin/NF2, Kibra, Lgl, and Melted specifies and maintains postmitotic neuronal fate. *Dev. Cell* **21**, 874–887 (2011). doi: [10.1016/j.devcel.2011.10.004](#); pmid: [22055343](#)
5. R. C. Hardie, in *Progress in Sensory Physiology*, D. Ottoson, Ed. (Springer, Berlin, 1985), vol. 5, pp. 1–79.
6. J. Rister, C. Desplan, D. Vasilakias, Establishing and maintaining gene expression patterns: Insights from sensory receptor patterning. *Development* **140**, 493–503 (2013). doi: [10.1242/dev.079095](#); pmid: [23293281](#)
7. Y. Goulev *et al.*, SCALLOPED interacts with YORKIE, the nuclear effector of the hippo tumor-suppressor pathway in *Drosophila*. *Curr. Biol.* **18**, 435–441 (2008). doi: [10.1016/j.cub.2008.02.034](#); pmid: [18313299](#)
8. L. Zhang *et al.*, The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control. *Dev. Cell* **14**, 377–387 (2008). doi: [10.1016/j.devcel.2008.01.006](#); pmid: [18258485](#)
9. S. Wu, Y. Liu, Y. Zheng, J. Dong, D. Pan, The TEAD/TEF family protein Scalloped mediates transcriptional output of the Hippo growth-regulatory pathway. *Dev. Cell* **14**, 388–398 (2008). doi: [10.1016/j.devcel.2008.01.007](#); pmid: [18258486](#)
10. H. W. Peng, M. Slattery, R. S. Mann, Transcription factor choice in the Hippo signaling pathway: Homothorax and yorkie regulation of the microRNA bantam in the progenitor domain of the *Drosophila* eye imaginal disc. *Genes Dev.* **23**, 2307–2319 (2009). doi: [10.1101/gad.1820009](#); pmid: [19762509](#)
11. H. Oh, K. D. Irvine, Cooperative regulation of growth by Yorkie and Mad through bantam. *Dev. Cell* **20**, 109–122 (2011). doi: [10.1016/j.devcel.2010.12.002](#); pmid: [21238929](#)
12. F. Hamaratoglu *et al.*, The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis. *Nat. Cell Biol.* **8**, 27–36 (2006). doi: [10.1038/ncb1339](#); pmid: [16341207](#)
13. A. Genevet, M. C. Wehr, R. Brain, B. J. Thompson, N. Tapon, Kibra is a regulator of the Salvador/Warts/Hippo signaling network. *Dev. Cell* **18**, 300–308 (2010). doi: [10.1016/j.devcel.2009.12.011](#); pmid: [20159599](#)
14. R. M. Neto-Silva, S. de Beco, L. A. Johnston, Evidence for a growth-stabilizing regulatory feedback mechanism between Myc and Yorkie, the *Drosophila* homolog of Yap. *Dev. Cell* **19**, 507–520 (2010). doi: [10.1016/j.devcel.2010.09.009](#); pmid: [20951343](#)
15. A. Swaroop, D. Kim, D. Forrest, Transcriptional regulation of photoreceptor development and homeostasis in the mammalian retina. *Nat. Rev. Neurosci.* **11**, 563–576 (2010). doi: [10.1038/nrn2880](#); pmid: [20648062](#)
16. J. Huang, S. Wu, J. Barrera, K. Matthews, D. Pan, The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the *Drosophila* Homolog of YAP. *Cell* **122**, 421–434 (2005). doi: [10.1016/j.cell.2005.06.007](#); pmid: [16096061](#)
17. J. Dong *et al.*, Elucidation of a universal size-control mechanism in *Drosophila* and mammals. *Cell* **130**, 1120–1133 (2007). doi: [10.1016/j.cell.2007.07.019](#); pmid: [17889654](#)
18. H. Oh, K. D. Irvine, In vivo regulation of Yorkie phosphorylation and localization. *Development* **135**, 1081–1088 (2008). doi: [10.1242/dev.015255](#); pmid: [18256197](#)
19. M. F. Wernet *et al.*, Homothorax switches function of *Drosophila* photoreceptors from color to polarized light sensors. *Cell* **115**, 267–279 (2003). doi: [10.1016/S0092-8674\(03\)00848-1](#); pmid: [14636555](#)
20. B. Zhao *et al.*, Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev.* **21**, 2747–2761 (2007). doi: [10.1101/gad.1602907](#); pmid: [17974916](#)
21. C. J. Evans *et al.*, G-TRACE: Rapid Gal4-based cell lineage analysis in *Drosophila*. *Nat. Methods* **6**, 603–605 (2009). doi: [10.1038/nmeth.1356](#); pmid: [19633663](#)
22. A. A. Teleman, Y.-W. Chen, S. M. Cohen, *Drosophila* Melted modulates FOXO and TOR activity. *Dev. Cell* **9**, 271–281 (2005). doi: [10.1016/j.devcel.2005.07.004](#); pmid: [16054033](#)
23. E. R. Vandendries, D. Johnson, R. Reinke, Orthodenticle is required for photoreceptor cell development in the *Drosophila* eye. *Dev. Biol.* **173**, 243–255 (1996). doi: [10.1006/dbio.1996.0020](#); pmid: [8575625](#)
24. A. Tahayato *et al.*, Otd/Crx, a dual regulator for the specification of ommatidia subtypes in the *Drosophila* retina. *Dev. Cell* **5**, 391–402 (2003). doi: [10.1016/S1534-5807\(03\)00239-9](#); pmid: [12967559](#)
25. B. Xie, M. Charlton-Perkins, E. McDonald, B. Gebelein, T. Cook, Senseless functions as a molecular switch for color photoreceptor differentiation in *Drosophila*. *Development* **134**, 4243–4253 (2007). doi: [10.1242/dev.012781](#); pmid: [17978002](#)
26. E. C. McDonald *et al.*, Separable transcriptional regulatory domains within Otd control photoreceptor terminal differentiation events. *Dev. Biol.* **347**, 122–132 (2010). doi: [10.1016/j.ydbio.2010.08.016](#); pmid: [20732315](#)
27. M. A. Li, J. D. Alls, R. M. Avancini, K. Koo, D. Godt, The large Maf factor Traffic Jam controls gonad morphogenesis in *Drosophila*. *Nat. Cell Biol.* **5**, 994–1000 (2003). doi: [10.1038/ncb1058](#); pmid: [14578908](#)
28. K. Kataoka, Multiple mechanisms and functions of maf transcription factors in the regulation of tissue-specific genes. *J. Biochem.* **141**, 775–781 (2007). doi: [10.1093/jb/mvm105](#); pmid: [17569705](#)
29. A. Swaroop *et al.*, A conserved retina-specific gene encodes a basic motif/leucine zipper domain. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 266–270 (1992). doi: [10.1073/pnas.89.1.266](#); pmid: [1729696](#)
30. A. J. Mears *et al.*, Nrl is required for rod photoreceptor development. *Nat. Genet.* **29**, 447–452 (2001). doi: [10.1038/ng774](#); pmid: [11694879](#)
31. H. Hao *et al.*, Transcriptional regulation of rod photoreceptor homeostasis revealed by in vivo Nrl targetome analysis. *PLOS Genet.* **8**, e1002649 (2012). doi: [10.1371/journal.pgen.1002649](#); pmid: [22511886](#)
32. B. J. Frankfort, R. Nolo, Z. Zhang, H. Bellen, G. Mardon, Senseless repression of rough is required for R8 photoreceptor differentiation in the developing *Drosophila* eye. *Neuron* **32**, 403–414 (2001). doi: [10.1016/S0896-6273\(01\)00480-9](#); pmid: [11709152](#)
33. M. Morey *et al.*, Coordinate control of synaptic-layer specificity and rhodopsins in photoreceptor neurons. *Nature* **456**, 795–799 (2008). doi: [10.1038/nature07419](#); pmid: [18978774](#)
34. M. Mishra *et al.*, Pph13 and orthodenticle define a dual regulatory pathway for photoreceptor cell morphogenesis and function. *Development* **137**, 2895–2904 (2010). doi: [10.1242/dev.051722](#); pmid: [20667913](#)
35. A. B. Hall *et al.*, hSpry2 is targeted to the ubiquitin-dependent proteasome pathway by c-Cbl. *Curr. Biol.* **13**, 308–314 (2003). doi: [10.1016/S0960-9822\(03\)00086-1](#); pmid: [12593796](#)
36. C. Rubin *et al.*, Sprouty fine-tunes EGF signaling through interlinked positive and negative feedback loops. *Curr. Biol.* **13**, 297–307 (2003). doi: [10.1016/S0960-9822\(03\)00053-8](#); pmid: [12593795](#)
37. M. Overholtzer *et al.*, Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 12405–12410 (2006). doi: [10.1073/pnas.0605579103](#); pmid: [16894141](#)
38. L. Zender *et al.*, Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. *Cell* **125**, 1253–1267 (2006). doi: [10.1016/j.cell.2006.05.030](#); pmid: [16814713](#)
39. B. Zhao, L. Li, Q. Lei, K.-L. Guan, The Hippo-YAP pathway in organ size control and tumorigenesis: An updated version. *Genes Dev.* **24**, 862–874 (2010). doi: [10.1101/gad.1909210](#); pmid: [20439427](#)
40. A. Eychène, N. Rocques, C. Poupponnot, A new MAFia in cancer. *Nat. Rev. Cancer* **8**, 683–693 (2008). doi: [10.1038/nrc2460](#); pmid: [19143053](#)
41. J. Bunt *et al.*, OTX2 directly activates cell cycle genes and inhibits differentiation in medulloblastoma cells. *Int. J. Cancer* **131**, E21–E32 (2012). doi: [10.1002/ijc.26474](#); pmid: [21964830](#)
42. W. Xiong, J. E. Ferrell Jr., A positive-feedback-based bistable 'memory module' that governs a cell fate decision. *Nature* **426**, 460–465 (2003). doi: [10.1038/nature02089](#); pmid: [14647386](#)
43. J. R. Pomeroy, E. D. Sontag, J. E. Ferrell Jr., Building a cell cycle oscillator: Hysteresis and bistability in the activation of Cdc2. *Nat. Cell Biol.* **5**, 346–351 (2003). doi: [10.1038/ncb954](#); pmid: [12629549](#)
44. J. M. Skotheim, S. Di Talia, E. D. Siggia, F. R. Cross, Positive feedback of G1 cyclins ensures coherent cell cycle entry. *Nature* **454**, 291–296 (2008). doi: [10.1038/nature07118](#); pmid: [18633409](#)
45. See supplementary materials on Science Online.

Acknowledgments: We thank J. Bell, S. Britt, R. Carthew, S. Cohen, I. Davis, B. Dickson, R. Fehon, D. Godt, G. Halder, I. Hariharan, K. Irvine, J. Jiang, D. J. Pan, N. Tapon, J. Treisman, T. Xu, C. Zuker, the Bloomington Stock Center, the Kyoto Stock Center, the Vienna *Drosophila* RNAi Center, and the Exelixis Collection at Harvard Medical School for providing fly stocks and antibodies. G. Mardon generously provided *sens-Gal4* flies and S. Sprecher kindly shared Pph13 results prior to publication. We thank T. Blackman and C. Tsanis for transgenic injections and members of the Desplan and Cook labs for discussions and comments. Supported by a New York University (NYU) Dean's Dissertation Award (D.J.), a University of Cincinnati Postdoctoral Research Fellowship (B.X.), European Molecular Biology Organization (EMBO) long-term fellowships (ALTF 506-2002 and ALTF 462-2008) (D.P. and J.R.), NIH grants RO1 EY13012 (C.D.) and RO1-EY017907 (T.C.), and Research to Prevent Blindness (T.C.).

Supplementary Materials
www.sciencemag.org/content/342/6155/1238016/suppl/DC1
 Materials and Methods
 Figs. S1 to S13
 References (46–65)

19 March 2013; accepted 29 July 2013
 Published online 29 August 2013;
[10.1126/science.1238016](https://doi.org/10.1126/science.1238016)

Villification: How the Gut Gets Its Villi

Amy E. Shyer,^{1*} Tuomas Tallinen,^{2,3*} Nandan L. Nerurkar,¹ Zhiyan Wei,² Eun Seok Gil,⁴ David L. Kaplan,⁴ Clifford J. Tabin,^{1†} L. Mahadevan^{2,5,6,7,8†}

The villi of the human and chick gut are formed in similar stepwise progressions, wherein the mesenchyme and attached epithelium first fold into longitudinal ridges, then a zigzag pattern, and lastly individual villi. We find that these steps of villification depend on the sequential differentiation of the distinct smooth muscle layers of the gut, which restrict the expansion of the growing endoderm and mesenchyme, generating compressive stresses that lead to their buckling and folding. A quantitative computational model, incorporating measured properties of the developing gut, recapitulates the morphological patterns seen during villification in a variety of species. These results provide a mechanistic understanding of the formation of these elaborations of the lining of the gut, essential for providing sufficient surface area for nutrient absorption.

In amniotes, the primitive midgut is established as a cylinder with an outer mesenchymal layer and an inner, luminal endoderm. As development proceeds, distinct radial layers of smooth muscle differentiate. In parallel, the luminal surface of the gut transforms from a smooth surface to a convoluted morphology. In humans, as well as in mice and birds, this leads to an organized array of fingerlike projections termed intestinal villi (1, 2) although a variety of morphologies such as

ridges, zigzags, and honeycombs occur in other species (3–5). Early work suggested a mechanical basis for villus formation (6); however, systematic biological or physical studies of this hypothesis are lacking.

Morphogenesis and Differentiation of the Chick Midgut

Until embryonic day 7 (E7), the gut tube, with its inner endodermally derived epithelium and outer

mesenchymal layer, maintains a smooth luminal surface (Fig. 1A). At E8, as the first layer of circumferentially oriented smooth muscle begins to form, inward buckling of the tube leads to longitudinal ridges that increase in number until E13, when the differentiation of this layer is complete (Fig. 1B). At this point, a second longitudinally oriented layer of muscle differentiates just exterior to the circular layer, while the previously formed ridges fold into parallel zigzags over 3 days (Fig. 1C). Last, at E16, as a third longitudinally oriented muscle layer differentiates just interior to the circular layer, bulges arise from the

¹Department of Genetics, Harvard Medical School, Boston, MA 02115, USA. ²School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138, USA. ³Department of Physics and Nanoscience Center, University of Jyväskylä, FI-40014 Jyväskylä, Finland. ⁴Department of Biomedical Engineering, Tufts University, Medford, MA 02155, USA. ⁵Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA. ⁶Department of Physics, Harvard University, Cambridge, MA 02138, USA. ⁷Wyss Institute for Biologically Inspired Engineering, Harvard University, Cambridge, MA 02138, USA. ⁸Kavli Institute for Nanobio Science and Technology, Harvard University, Cambridge, MA 02138, USA.

*These authors contributed equally to this work.

†Corresponding author. E-mail: lm@seas.harvard.edu (L.M.); tabin@genetics.med.harvard.edu (C.J.T.)

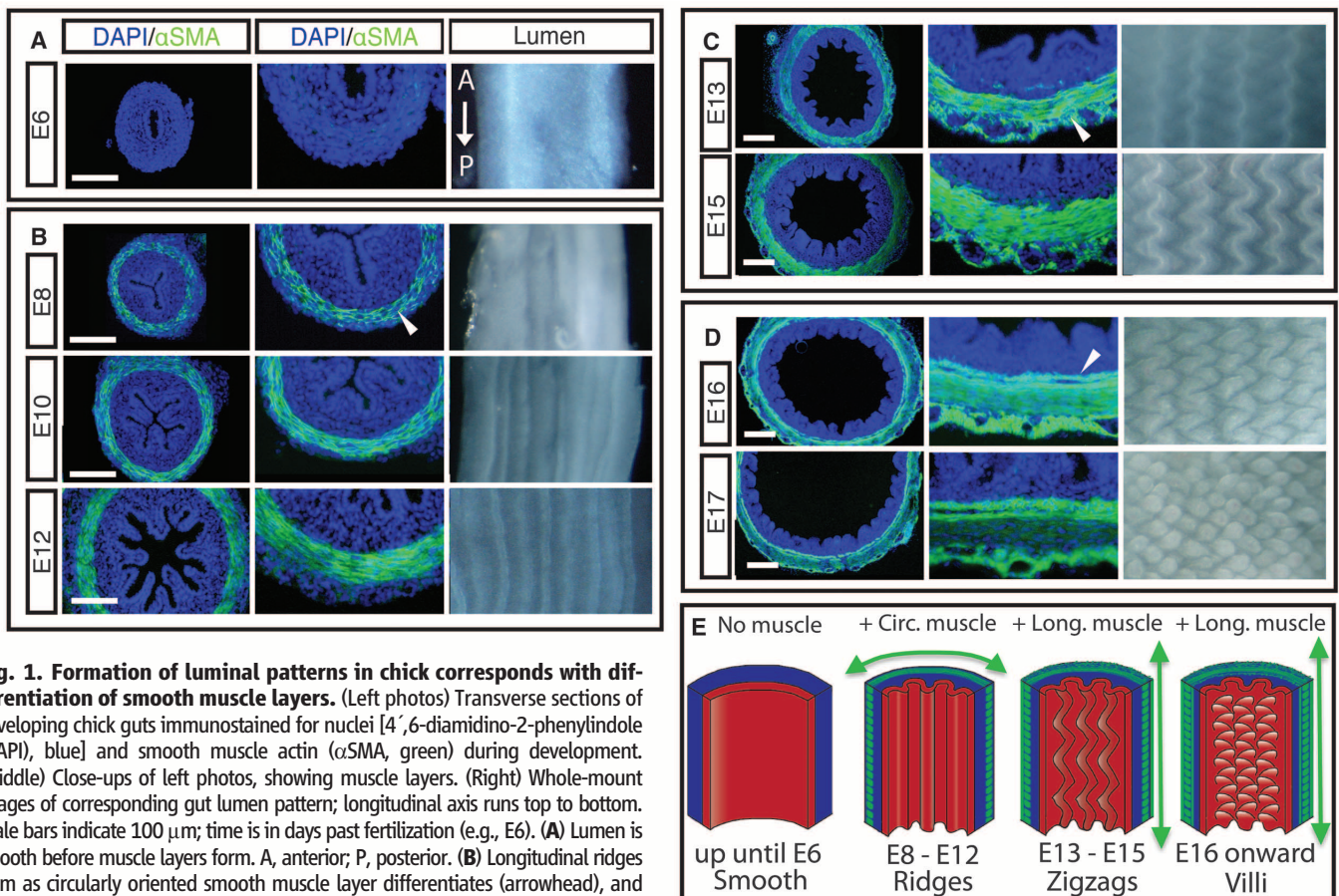


Fig. 1. Formation of luminal patterns in chick corresponds with differentiation of smooth muscle layers. (Left photos) Transverse sections of developing chick guts immunostained for nuclei [4',6-diamidino-2-phenylindole (DAPI), blue] and smooth muscle actin (α SMA, green) during development. (Middle) Close-ups of left photos, showing muscle layers. (Right) Whole-mount images of corresponding gut lumen pattern; longitudinal axis runs top to bottom. Scale bars indicate 100 μ m; time is in days past fertilization (e.g., E6). (A) Lumen is smooth before muscle layers form. A, anterior; P, posterior. (B) Longitudinal ridges form as circularly oriented smooth muscle layer differentiates (arrowhead), and ridge number increases as this layer develops. (C) Longitudinal muscle develops exterior to the circular layer (arrowhead) coincident with the formation of zigzags whose periodicity is maintained but with increasing amplitude and compactness over time. (D) A second longitudinal muscle layer forms, interior to the circular layer (arrowhead), coincident with the formation of villi. (E) Schematic illustrating the process of muscle differentiation and luminal patterning over time.

zigzag pattern that presage the formation of villi (Fig. 1D). The coincident emergence of luminal ridges, zigzags, and villi with the sequential formation of smooth muscle layers suggests that smooth muscle differentiation and epithelial morphogenesis might be linked.

Ridges Form Because of Muscle-Constrained Azimuthal Growth of the Endoderm-Mesenchyme Composite

The notion that differential growth of layered tissues can lead to epithelial buckling is classical (7, 8) and has been evoked, for example, to explain longitudinal ridge formation in healthy and diseased adult trachea and esophagus (4, 9). To investigate the tissue interactions that lead to the ridge patterns in the embryonic gut, we surgically separated the layers and observed the effects on their respective morphologies. When the muscle was separated from the combined mesenchymal and epithelial layers at different stages from E8, when the circular muscle layer first forms, to E12 just before the first longitudinal muscle layer forms, we found that the mesenchyme and attached epithelium unfold (Fig. 2A). This indicates that relative growth of these layers leads to reversible elastic compression when constrained within the muscle layer; indeed the ratio of the inner circumference of the once-attached muscle layer to the outer circumference of the separated mesenchyme and endoderm, the circumferential stretch ratio, consistently averages to 0.55 across the developmental stages from E8 to E12 (Fig. 2B). However, the separation of the endoderm from the composite of mesenchyme and muscle does not abolish ridge pattern in the mesenchyme (Fig. 2C).

Taken together, these results support a model that the circular muscle layer, once differentiated, forms a stiff constraint mechanically preventing the free azimuthal expansion of the mesenchyme and endoderm; further growth of these tissues relative to the muscle layer leads to azimuthal compression and buckling. This suggests that absent muscle differentiation, the gut tube would expand freely radially without ridge formation. To test this, we developed an in vitro culture system for gut growth. When segments of E6 guts with smooth lumens and no muscle layers were cultured for 48 hours in vitro, they differentiated to form a ring of circumferential smooth muscle and parallel luminal folds, indistinguishable from in ovo E8 guts (Fig. 2D). When E6 guts were cultured in the presence of 10 μ M AG1295 or FK506, drugs known to block the differentiation of smooth muscle but that act through distinct signaling pathways (10, 11), they did not form a smooth muscle layer and concomitantly did not form luminal folds (Fig. 2D). Importantly, these compounds did not influence proliferation or lead to an increase in cell death when compared with guts grown with the vehicle (dimethyl sulfoxide, DMSO) alone (fig. S1); indeed there was a significant increase in the outer circumference of guts lacking circular smooth muscle when com-

pared with control gut samples, confirming that blocking smooth muscle differentiation eliminates circumferential restriction of the outward expansion of the gut tube. As a control, gut segments grown in vehicle alone developed a layer of circular smooth muscle and formed luminal folds. Quantifying the constraint provided by the muscle, we find that the ratio of inner circumference of the muscle layer in the control samples to the outer circumference of the gut segments cultured

with either compound to be 0.53 on average (Fig. 2D), a ratio that agrees closely with the stretch ratio obtained from surgical separation of the layers, indicating that tissue differentiation into smooth muscle provides most of the circumferential constraint.

Because smooth muscle begins active peristalsis once it forms, the contractility of muscle could drive epithelial buckling in addition to, or instead of, functioning as a passive barrier to expansion. To test this, we cultured E6 gut segments

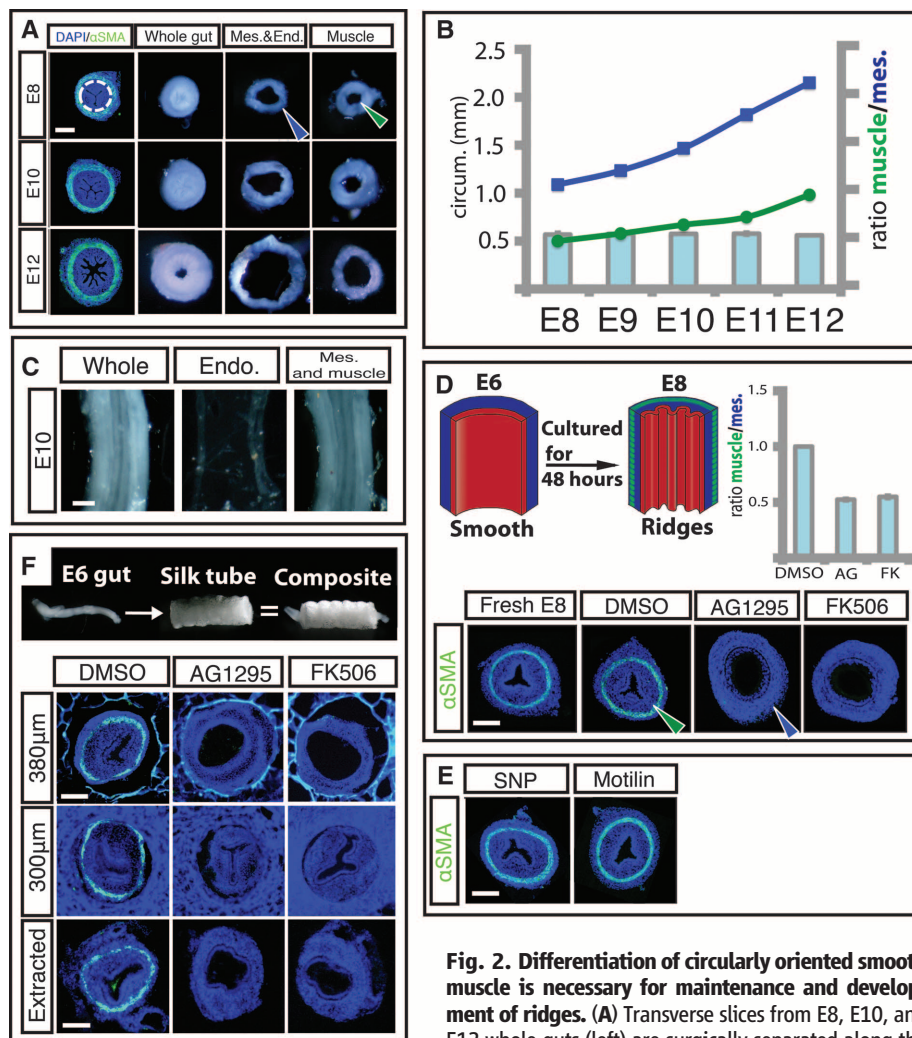


Fig. 2. Differentiation of circularly oriented smooth muscle is necessary for maintenance and development of ridges. (A) Transverse slices from E8, E10, and E12 whole guts (left) are surgically separated along the junction of the mesenchyme and the circular smooth

muscle (dotted line). When separated from the muscle, the luminal ridges in the mesenchyme and attached endoderm unfold (middle) and expand, whereas the detached muscle remains invariant (right). The outer circumference of the unfolded mesenchyme and endoderm (blue arrowhead) is larger than the inner circumference of the separated muscle layer (green arrowhead). (B) Inner circumference of muscle layer (green line) compared with outer circumference of mesenchyme and endoderm (blue line) over time, along with the compression ratio (bar graph). (C) Surgical separation of endoderm from mesenchyme and muscle at E10 does not abolish ridge pattern. (D) (Top left) Experiment schematic of E6 gut cultured for 48 hours. (Bottom) Transverse sections of a fresh E8 gut or E6 guts cultured in DMSO alone or with either 10 μ M AG1295 or 10 μ M FK506 for 48 hours and labeled with DAPI (blue) and SMA (green). (Top right) Quantification of compression from E8 muscle shows the ratio of the inner circumference of the circular muscle at E8 (green arrowhead) to the resulting mesenchyme outer circumference (blue arrowhead). (E) Transverse sections of guts labeled as in (D); culturing E6 guts in the presence of either SNP or motilin does not affect ridge formation. (F) Transverse sections of guts labeled as in (D), cultured in silk tubes of 380- μ m inner diameter (top) or 300- μ m inner diameter (middle) or cultured in 300 μ m and extracted before fixation (bottom). $n > 3$ for all culture experiments; error bars represent one SD. Scale bars = 100 μ m.

with either sodium nitroprusside (SNP), a compound shown to inhibit smooth muscle contraction during peristalsis, or motilin, known to enhance peristaltic smooth muscle contraction (12, 13). After 48 hours in culture, neither compound affected the formation of luminal ridges, suggesting that the spontaneous contractility of smooth muscle is not required for epithelial buckling (Fig. 2E).

Last, to assess whether the role of the circular smooth muscle layer as a stiff barrier is sufficient to drive luminal folds, we sought to mimic its role in samples where smooth muscle development was blocked. To do so, we constrained radial gut growth by using porous silk tubes, synthesized by spinning silk fibroin around a reciprocating rotating mandrel with the inner diameter of the circular smooth muscle (14). E6 gut segments cultured inside of silk tubes in the presence of either AG1295 or FK506 for 48 hours did not form a muscle layer and, when given sufficient room to expand in silk tubes of 380- μ m inner diameter, still did not form luminal ridges (Fig. 2F). However, when the segments were grown in AG1295 and FK506 and restricted by a silk tube of inner diameter of 300 μ m, they formed ridges similar to those seen in control guts in spite of the lack of smooth muscle (Fig. 2F). This demonstrates that the mechanical barrier function of the circumferential smooth muscle is sufficient for luminal ridge formation. Moreover, upon removal from the confining silk tube, these ridges were quickly lost, just as they vanished from gut tubes upon surgical removal of the circumferential muscle layer (Fig. 2F), corroborating our previous finding that continued mechanical constraint is required for the maintenance of luminal ridges.

Zigzag Intermediates Form in Response to Additional Muscle-Constrained Longitudinal Growth in Endoderm-Mesenchyme Composite

As described earlier, at E13 a second longitudinally oriented muscle layer forms; simultaneously the ridges buckle into zigzags. Previous work has shown that a thin layer atop an elastic substrate may take on a zigzag topography when it is compressed biaxially (15–17), suggesting that the longitudinal muscles in conjunction with the previously established circumferential muscle compress the gut biaxially. To investigate whether the longitudinal muscle layer generates longitudinal compression, we surgically separated the muscle layers from the endoderm-mesenchyme composite at different developmental stages. At E12, before longitudinal muscle or zigzags have formed, the separated mesenchyme and attached endoderm have about the same axial length as the muscle to which they were attached (Fig. 3A). However, after longitudinal muscle layer differentiation, at E13, E14, and E15, the ratios of the length of separated muscle to the mesenchyme and endoderm were about 0.75, 0.69, and 0.55, respectively (Fig. 3A), showing that the endoderm-mesenchyme is increasingly compressed longitudinally as this muscle layer forms. Conversely, separation of the endoderm from the

mesenchyme and muscle at E14 did not abolish the zigzag pattern, suggesting that this interaction is not required for maintenance of the zigzags (Fig. 3B).

To directly test whether the development of the outer longitudinal layer is required for the formation of zigzags, we resorted again to our *in vitro* culture system. When E12 gut segments, with a single, circumferential smooth muscle layer and parallel ridges, were cultured for 48 hours, they differentiated a longitudinal smooth muscle layer and underwent morphogenesis to form zigzags, similar to those seen in guts harvested at E14 (Fig. 3C). In the presence of either AG1295

or FK506, the longitudinal muscle layer failed to differentiate, and concomitantly the zigzag pattern did not form, suggesting that the longitudinal layer is required to induce zigzags. These compounds only block further smooth muscle formation and leave established layers intact, so the ridge patterns remain (Fig. 3C). Additionally, when differentiation of this longitudinal muscle was blocked, the length of the gut increased significantly compared with control gut segments; the ratio of the length of control gut segments to those cultured in the presence of either compound was on average 0.66; that is, this longitudinal muscle layer compressed the mesenchyme and attached

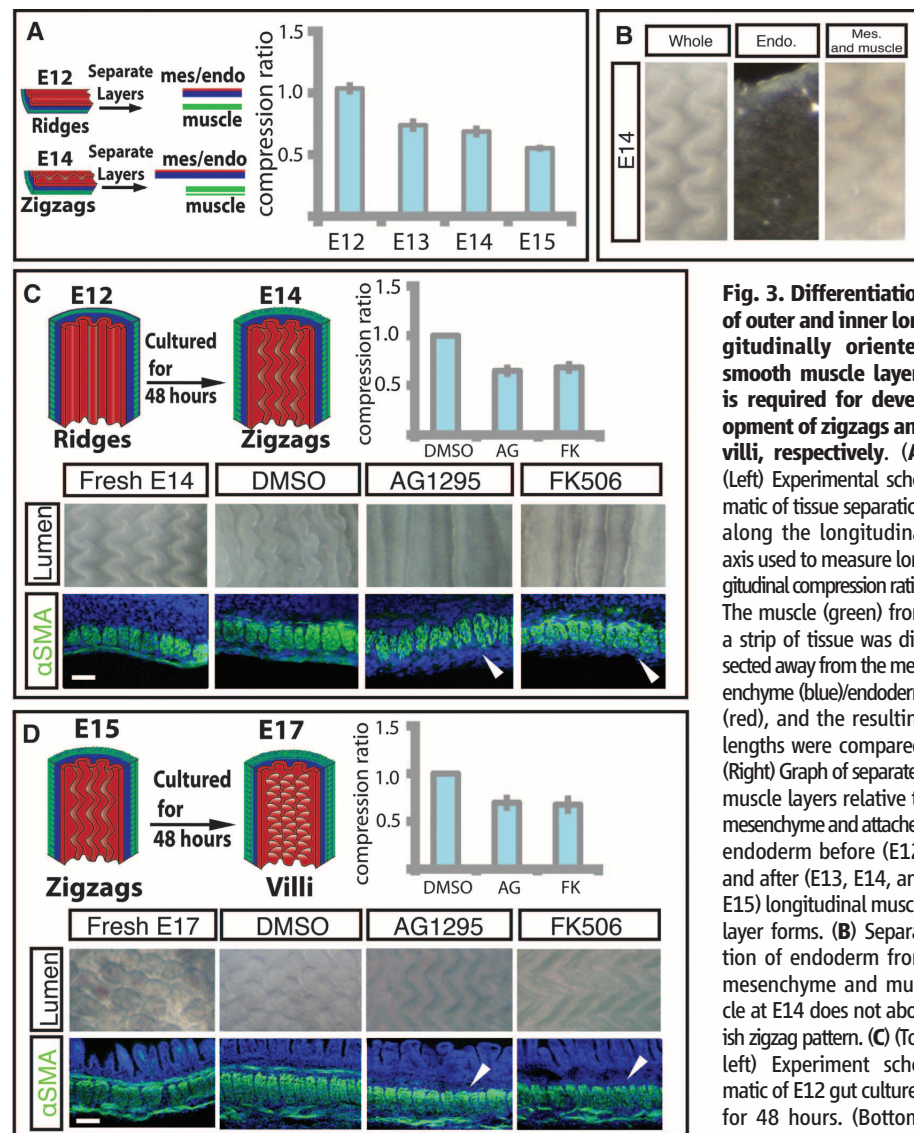


Fig. 3. Differentiation of outer and inner longitudinally oriented smooth muscle layers is required for development of zigzags and villi, respectively. (A) (Left) Experimental schematic of tissue separation along the longitudinal axis used to measure longitudinal compression ratio. The muscle (green) from a strip of tissue was dissected away from the mesenchyme (blue)/endoderm (red), and the resulting lengths were compared. (Right) Graph of separated muscle layers relative to mesenchyme and attached endoderm before (E12) and after (E13, E14, and E15) longitudinal muscle layer forms. (B) Separation of endoderm from mesenchyme and muscle at E14 does not abolish zigzag pattern. (C) (Top left) Experiment schematic of E12 gut cultured for 48 hours. (Bottom) E12 guts cultured in DMSO

alone or with either 10 μ M AG1295 or 10 μ M FK506 for 48 hours. Middle photos show luminal views, and bottom photos show longitudinal sections labeled with DAPI (blue) and SMA (green). Arrowheads denote absence of muscle layer. (Top right) Quantification of compression from E14 longitudinal muscle characterized by the ratio of the length of the control cultured segments to those lacking muscle. (D) (Top left) Experiment schematic of E15 gut cultured for 48 hours. (Bottom) Fresh E17 gut or E15 guts cultured in DMSO alone or with either 10 μ M AG1295 or 10 μ M FK506 for 48 hours. Middle photos show luminal views and bottom photos show longitudinal sections, labeled as in (C). Arrowheads denote absence of muscle layer. (Top right) Quantification of compression from E16 longitudinal muscle, as in (C). $n > 3$ for all culture experiments; error bars represent one SD. Scale bars = 20 μ m.

epithelium by a factor of about 1.5 (Fig. 3C). This is consistent with the value obtained by manual dissection of the layers. Just as for ridge formation, transformation of ridges to zigzags is independent of smooth muscle contractility (fig. S2).

Villification Requires a Third Regime of Smooth Muscle Differentiation

To investigate the dependence of the final patterning of villi on the differentiation of the inner, longitudinal smooth muscle layer, we cultured E15 guts, with both a circumferential layer and outer longitudinal layer, for 48 hours in the presence of the muscle-blocking compounds or with the vehicle (DMSO) alone. Although gut segments cultured with DMSO developed a third inner longitudinal muscle layer and formed villi, those cultured with either AG1295 or FK506 failed to form this muscle layer and did not initiate villi (Fig. 3D). When differentiation of this longitudinal muscle was blocked, the length of the tube increased significantly compared with those of control gut segments (Fig. 3D). The ratio of the length of control gut segments to that of those lacking the outer longitudinal muscle was on average 0.68; that is, this muscle layer compressed the mesenchyme and endoderm again by a factor of about 1.5 (Fig. 3D).

All together, our surgical manipulations and drug studies support the hypothesis that differentiating smooth muscle acts as a barrier to the expansion of the attached mesenchyme and endoderm, compressing these layers first circumferentially to form ridges, then longitudinally to form zigzags, and last longitudinally again to form villi. We emphasize that, because the patterns relax when the muscular constraints are released surgically, it follows that the morphology of the lumen is a simple consequence of elastic energy minimization during the constrained growth of a soft, layered elastic tissue.

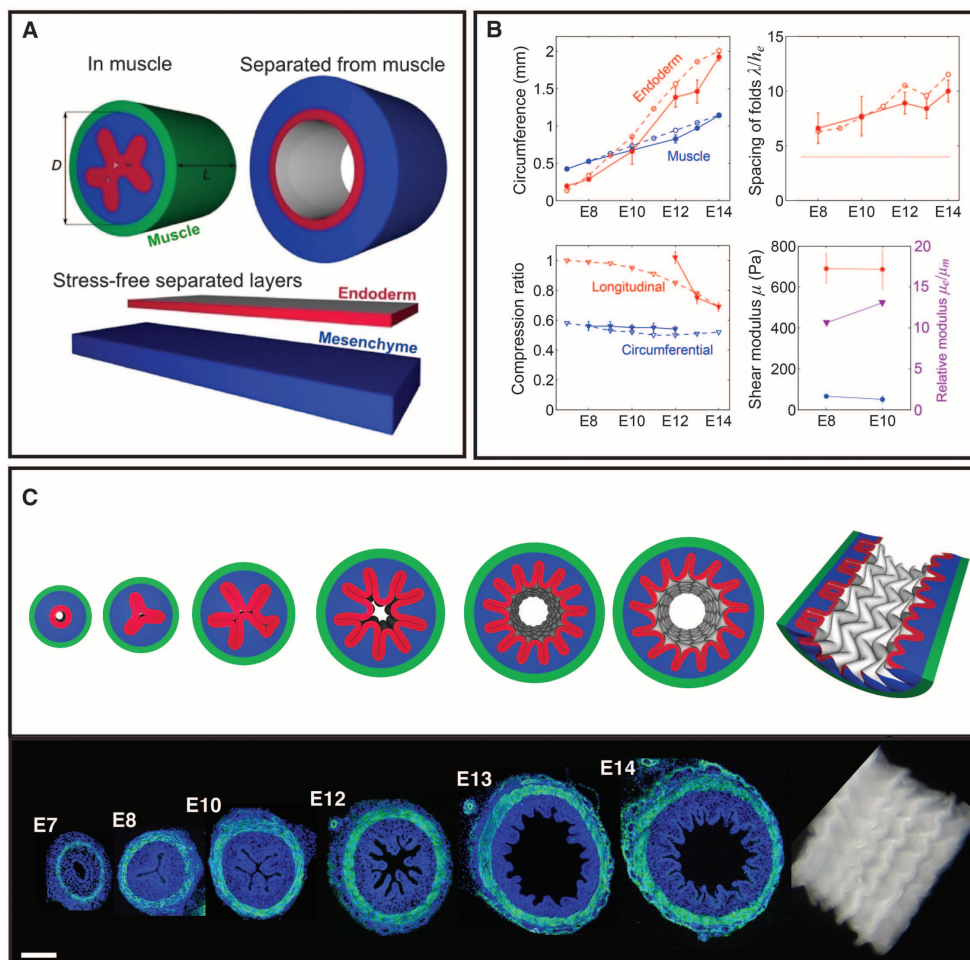
Mathematical Models Quantify the Role of Tissue Growth Constrained by Muscle Layers to Drive Ridge and Zigzag Formation

To further quantify these luminal patterns, we constructed a mathematical and computational model of the process based on measured geometrical and biophysical parameters. Our models are similar in spirit to recent theoretical approaches to gut luminal patterning based on the hypothesis of differential growth (18, 19) but go beyond them by correctly accounting for the constraints provided by the combination of muscular differentiation and differential growth that we see evidence for, the cylindrical geometry of the gut, and the

experimentally measured geometrical and physical properties of the system.

We start by considering a composite of naturally flat elastic mesenchyme and endoderm sheets that are attached together and bent and squeezed to fit into a rigid tubular configuration of inner diameter D that mimics the circular muscle layer (Fig. 4A). We assume that the tissues may be well described by using a simple neo-Hookean constitutive model, with a volumetric strain energy density $W = \frac{1}{2}\mu[\text{Tr}(\mathbf{F}\mathbf{F}^T)J^{-2/3} - 3] + K(J - \log J - 1)$, where μ and K are the shear and bulk moduli, respectively; $\mathbf{F}$ is the elastic deformation gradient; and $J = \det(\mathbf{F})$. Over the multiday time scale of villification, the tissues are assumed compressible with $K = 3\mu$. At each stage, we minimize the elastic energy of the system by using a custom finite element model (supplementary materials). We characterize growth by using the experimentally measured growth parameters, including the outer circumference, $S_0 = \pi D$, of the compressed endoderm-mesenchyme composite, as well as the circumference and thickness of the endoderm and mesenchyme (Fig. 4B and fig. S7). The simulated domain has length $L = 1.25D$ in the longitudinal direction with periodic boundary conditions at the ends, allowing uniform relative longitudinal growth of the layers to develop

Fig. 4. A numerical simulation predicts the formation of ridges and zigzags in chick gut lumen. (A) The model is illustrated by showing the mesenchyme (blue) and the endoderm (red) enclosed in a muscle (green), without muscle, and separated in their stress-free states. (B) (Top left) Circumference of the inner boundary of the muscle and endoderm. (Top right) Spacing of longitudinal folds measured along the endoderm and scaled by its thickness. The thin dashed line is the stress-free thickness of the endoderm-mesenchyme composite. (Bottom left) Ratio of muscle to separated endoderm-mesenchyme composite in circumferential and longitudinal directions. (Bottom right) Shear modulus of mesenchyme and endoderm, and their ratio. In all graphs, solid lines correspond to experimental observations and dashed lines to the computational model. Error bars, 1 SD. (C) A simulation shows ridge-folds forming due to circumferential compression, followed by buckling into a zigzag pattern due to longitudinal compression. Sections of corresponding chick guts labeled with DAPI (blue) and SMA (green) are shown below.



axial compression that mimics the role of the longitudinal muscles at E13 and E14 when zigzags arise. With the geometrical parameters (full details in the supplementary materials) and the measured elastic moduli of the tissues (Fig. 4B and figs. S3 to S6) that show that the endoderm is about 10 times stiffer than the mesenchyme, our simulations allow us to follow the evolution of luminal patterning shown in Fig. 4C and movie S1. We see that both ridge and zigzag patterns arise as mechanical instabilities in the constrained growing tissue that sequentially break circumferential and then longitudinal symmetry in the gut with a wavelength and amplitude comparable to the thickness of the endoderm-mesenchyme composite (Fig. 4B).

Villification Also Requires Localized Changes in Endodermal and Mesenchymal Proliferation in Addition to Smooth Muscle Differentiation

Although additional compression from the inner longitudinal layer is necessary for the formation of villi from zigzags, as shown in Fig. 3, longitudinal compression alone is not sufficient to effect this transformation (fig. S9A).

Previous work in mouse has shown that, although proliferating cells can be found uniformly across the mesenchyme and endoderm before villi arise, as villi form, proliferating cells are found only in the intervillous region (2). Similarly, in chick guts, proliferating cells appear uniformly within each tissue layer through the formation of zigzags (Fig. 5 and fig. S8), but at E15, after zigzags form and just before villi arise, proliferating cells are found predominantly in the valleys between the raised zigzags (Fig. 5A). However, once villi begin to form at E16, proliferation is no longer restricted from the tips (Fig. 5A). Additionally we find that *in vitro* 5-ethynyl-2'-deoxyuridine (EdU) pulse labeling of E15 gut samples results in labeled cells at the sides and tips of forming villi, suggesting that these changes in proliferation patterns may reflect a displacement of the dividing cells upward from the valleys as the luminal topography shifts from zigzags to villi. Specifically, each "arm" of the zigzag twists out of the plane and into the lumen, pinching off a region of the zigzag arm near each "elbow," delineating pockets of mesenchyme surrounded by endoderm, each of which becomes a villus (Fig. 5B).

To understand how the topographical changes during zigzag twisting might relocate regions of proliferation as villi form, we created a clay model of zigzags. Labeling the proliferating regions of our model zigzags and manually twisting them mimics the twist observed in the E16 gut (Fig. 5C). Furthermore, the resulting clay label localization closely matches EdU staining for proliferation in the sectioned E16 gut tissue (Fig. 5C), suggesting that these tissue movements account for the observed proliferation patterns as villi form.

To probe the effect of nonuniform growth in our computational model, we set up a minimal planar configuration of mesenchyme and endo-

derm (supplementary materials, fig. S9, and movie S2). Initially, the endoderm and mesenchyme are assumed to have nominal compression ratios of 0.5 and 0.6, respectively, in both lateral directions, as measured experimentally (Fig. 3A). This results in a tightly packed zigzag pattern (fig. S9A), with a spacing of twice the thickness of the endoderm-mesenchyme composite in both directions, in agreement with experiments. By using our experimental observations of nonuniform proliferation as guides, we incorporate non-uniform growth to this pattern by allowing the growth of spots of the endoderm in the zigzag

valleys, centered at the deepest points of the valleys, with lateral diameter six times the endoderm thickness. These spots are grown laterally until their diameter doubles during the simulation relative to areas of the endoderm outside the spots. This pattern of growth causes the zigzags to shift and twist so as to relocate the rapidly growing regions to the arms, similar to our clay model. As the spots relieve their growth strain at the arms, they form previllous bulges (Fig. 5E). Sliced plane views of this twisted pattern reveal their similarity to the corresponding experimental patterns (Fig. 5F); bulging peaks are rotated,

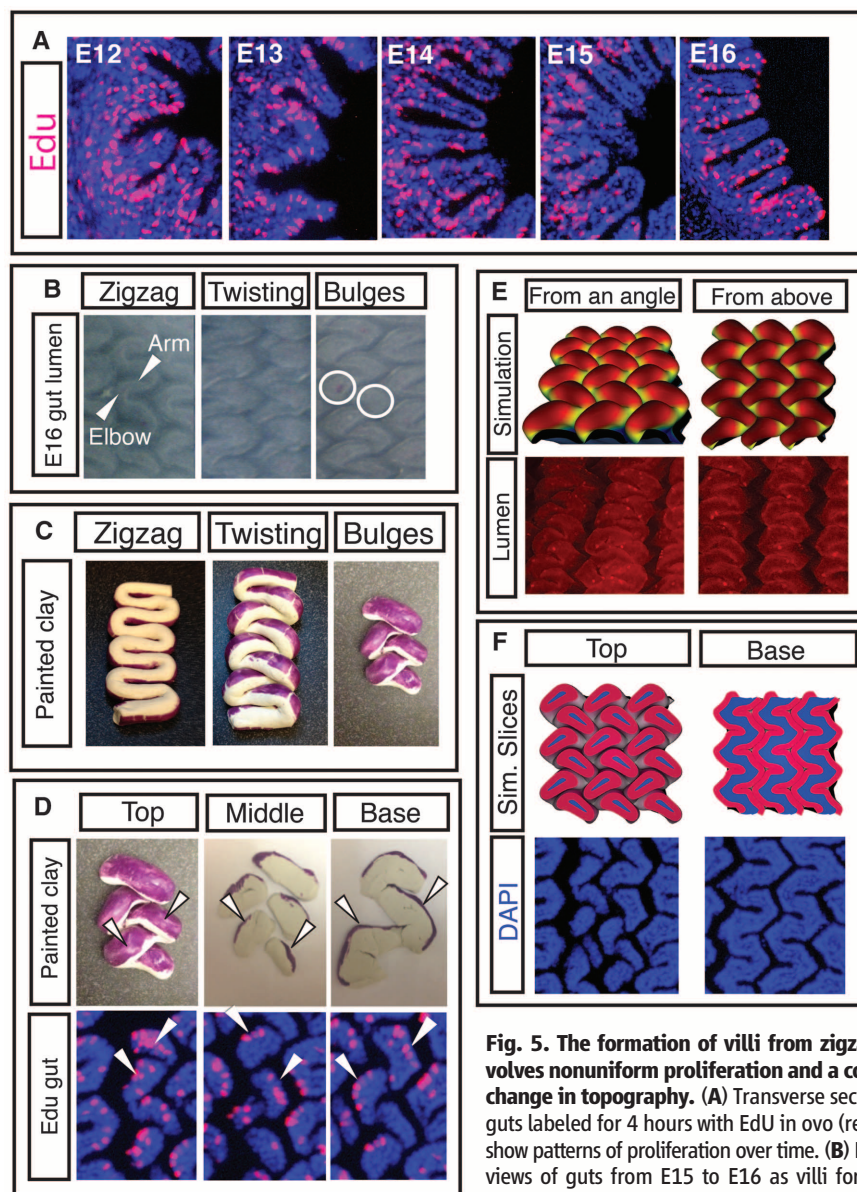


Fig. 5. The formation of villi from zigzags involves nonuniform proliferation and a complex change in topography. (A) Transverse sections of guts labeled for 4 hours with EdU in ovo (red) guts show patterns of proliferation over time. (B) Luminal views of guts from E15 to E16 as villi form. The "arm" of the zigzag rotates at the "elbow"; the circles denote the resulting pockets of mesenchyme surrounded by endoderm that will each become a villus. (C) Clay models; purple label represents proliferating regions. Clay model is twisted to mimic change in topography seen in (B). (D) (Top) Labeled, twisted model of E16 gut is sliced with a razor blade to reveal label localization. (Bottom) EdU label in longitudinal sections of E16 guts; arrowheads highlight similarity of pattern. (E) (Top) A simulation that incorporates nonuniform proliferation along with measured geometrical and biophysical parameters shows villi morphogenesis. (Bottom) Corresponding images of the chick lumen (red color and stained puncta are due to antibody stain and should be disregarded). (F) (Top) Sections of the simulations in (D). (Bottom) Corresponding sections in chick.

whereas the regular zigzag valleys persist deeper in the pattern.

Thus, although the final patterning step where definitive villi arise involves more complex morphogenesis and nonuniform proliferation, it follows from the same general physical principle: Differential growth in a constrained environment leads to buckling and folding patterns as circumferential, longitudinal, and eventually radial symmetry are broken sequentially.

A Phylogenetically Conserved Mechanism Directs Luminal Gut Morphogenesis

Although the patterns seen on the luminal surface of the gut vary substantially across species (fig. S10), the underlying physical principles we have uncovered for the chick lumen morphology suggest that similar mechanisms operate broadly.

In the adult *Xenopus*, the luminal surface of the intestine is folded into a zigzag pattern (4). Development of this pattern involves progressing through the same patterning steps as in chick, with a smooth lumen forming ridges that then develop into zigzags via identical mechanisms (Fig. 6A). However, *Xenopus*, unlike chick, does not develop the second inner longitudinal muscle layer (Fig. 6A); the absence of this muscular layer, and thence the absence of additional compression, explains why individual villi do not develop in *Xenopus*. Our computational models can account for the differences in zigzags between *Xenopus* and chick, as well as more exotic patterns seen in snakes (supplementary materials and fig. S10).

In the mouse, the gut does not progress through ridges and zigzags; instead, villi emerge directly from a smooth lumen (20). Although these villi arise only once smooth muscle layers form, the layers differentiate much more rapidly in mouse than in chick (Fig. 6E). This suggests that the relatively quick pace at which muscle layers form in the mouse does not leave time for proliferation and expansion of the inner mesenchyme and endoderm between the differentiation of sequential muscle layers and thus prevents the development of visible intermediate patterns such as ridges and zigzags. Specifically, all muscle layers develop within a 48-hour period, a short time compared with the 8 days required for muscle to fully develop in chick (20). To experimentally determine whether villus formation in mouse also requires differentiation of smooth muscle, we tested the effect of the smooth muscle inhibitors used in our chick studies on the formation of villi in mouse guts grown in culture. Just as in chick, the mouse guts grown in the presence of AG1295 or FK506 did not form smooth muscle and concomitantly did not develop villi (Fig. 6B), suggesting that compression from the smooth muscle layer is necessary for, and drives the formation of, villi in mouse.

Our studies are in sharp contrast to a recent view of mouse gut patterning that postulates a potential inductive role of the endodermally derived signal Sonic hedgehog (Shh) in triggering

a morphogenetic cascade directing villus outgrowth (21). The key results that led in this direction were the failure of villus formation when Shh activity was pharmacologically blocked with the Shh antagonist cyclopamine and the increased size of the villi when guts were provided with excess Shh signal. However, because these reagents were applied before villus formation, they were de facto also treated before smooth muscle differ-

entiation. Because Shh activity is both necessary and sufficient to direct smooth muscle formation in the developing intestine (22, 23), an alternative explanation would be that cyclopamine, by preventing smooth muscle specification, eliminates the constraint necessary for villi to form, consistent with our current studies.

To quantitatively test our theory of villification in the mouse gut, we performed mechanical

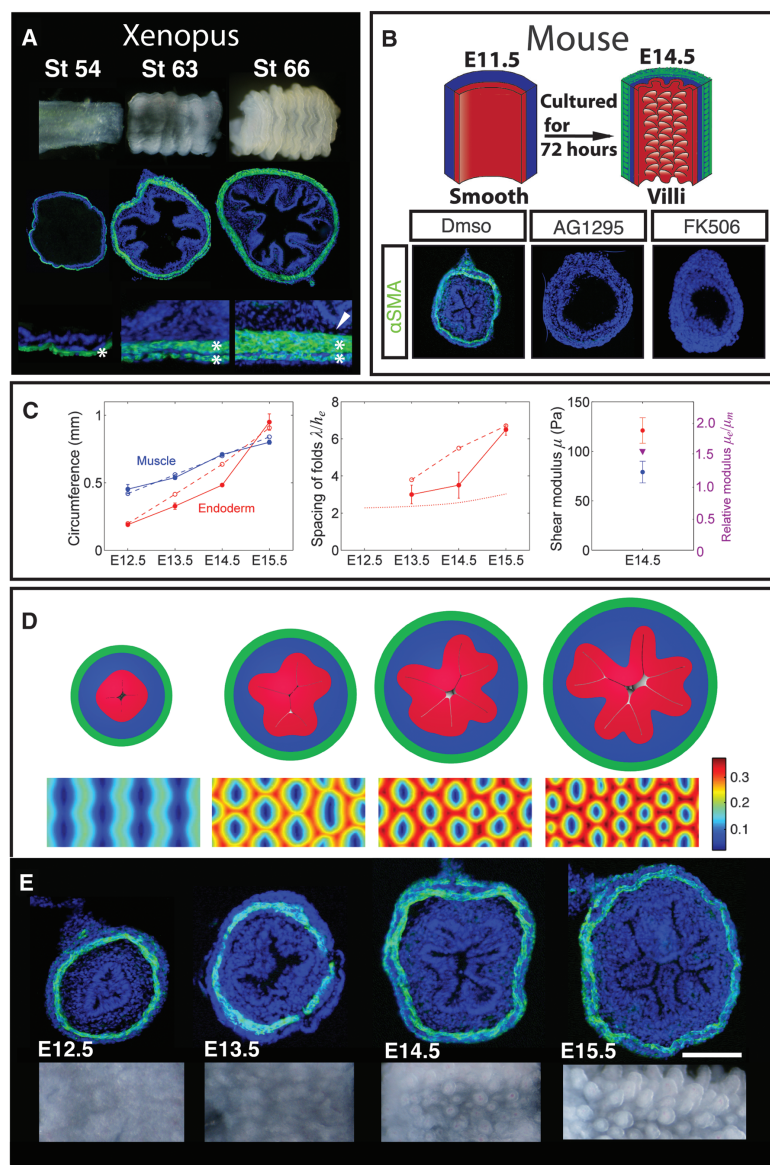


Fig. 6. The physical mechanism of villification can be extended to other species. (A) Luminal pattern formation in *Xenopus*. Sections labeled with DAPI (blue) and SMA (green). The circumferential and outer longitudinal layers form (asterisks), but the inner longitudinal layer does not form (arrowhead). (B) Transverse sections of E11.5 mouse guts [labeled as in (A)] cultured in vehicle alone (DMSO) or with either 10 μ M AG1295 or 10 μ M FK506 for 72 hours; experiment schematized above. (C) (Left) Circumference of the inner boundary of the muscle and endoderm. (Middle) Spacing of folds measured along the endoderm and scaled by its thickness. Dotted line is the stress-free thickness of the endoderm-mesenchyme composite. (Right) Shear moduli of mesenchyme and endoderm and their ratio. In all graphs, solid lines correspond to experimental observations and dashed lines to simulations. (D) Cross-sectional (top) and luminal (bottom) images from a simulation based on measurements from the developing mouse gut. Color shows distance of the luminal surface to the center line, relative to the diameter of the tube. (E) Transverse sections [labeled as in (A)] and whole-mount images of the lumen for corresponding stages during mouse villi formation. $n > 3$ for culture experiments; error bars represent one SD. Scale bars = 100 μ m.

and morphometric measurements of the tissues in the developing mouse gut (Fig. 6C). Using these measurements as inputs in our model suffices to quantitatively predict the formation of villi (supplementary materials, Fig. 6D, and movie S3). Compared with the chick, where the endoderm is more than 10 times stiffer than the adjacent mesenchyme, the mouse endoderm is only about 1.5 times as stiff as the mesenchyme (fig. S3). Our simulations show that the soft endoderm in mouse is essential for the initial folding that occurs in endoderm alone and for the direct formation of an array of previllous bumps, rather than zig-zags, which are qualitatively similar to sulcus formation on biaxially compressed gel surfaces that lack a stiff top layer (24). The spacing of bumps and, consequently, the spacing of villi are comparable to the thickness of the whole endoderm-mesenchyme composite (Fig. 6C), similar to chick.

The process of villification occurs before the differentiation of the gut endoderm into various epithelial cell types (25–27) and well before the postnatal process of crypt formation. In vitro culture of intestinal stem cells results in the formation of intestinal organoids that reproduce crypt structure (28). These organoids consist of an inner epithelium with villuslike cell types and outwardly projecting cryptlike structures. However, no morphological structures are present in these in vitro cultures resembling the physical villi. These results suggest that crypt formation likely does not require the same muscle-driven compression that is necessary for villi to form.

Additionally, further study is needed to understand whether structural differences in the lumen of different regions of the gut are attributable to distinctions in the parameters we have measured. For example, the short, wide villi that coat large longitudinal folds of the chick colon may be attributable to the thicker muscle layers of the colon. Consistent with the muscle playing

such a role, studies have shown that transposition of a ring containing all radial layers of the colon into regions of the small intestine preserve villi morphology (29).

Our previous work provided a mechanical basis for the diversity of macroscopic looping patterns of the gut based on geometry, differential growth, and tissue mechanics (30), and our present results demonstrate that the same physical principles drive morphological variation on the luminal surface of the gut. Further, we see that relatively minor changes in the geometry, growth, and physical properties of the developing tissue in the guts of various species can substantially alter both the process and the form of villus patterning. A deep understanding of how patterns vary requires us to combine our knowledge of biophysical mechanisms with the genetic control of cell proliferation and growth; indeed this variation can occur in an organism as a function of its diet, across species, and over evolutionary time scales via natural selection.

References and Notes

1. V. A. McClint, S. J. Henning, M. Jamrich, *Gastroenterology* **136**, 2074–2091 (2009).
2. T. K. Noah, B. Donahue, N. F. Shroyer, *Exp. Cell Res.* **317**, 2702–2710 (2011).
3. W. J. Krause, *Anat. Histol. Embryol.* **40**, 352–359 (2011).
4. J. W. McAvoy, K. E. Dixon, *J. Anat.* **125**, 155–169 (1978).
5. S. Ferri, L. C. U. Junqueira, L. F. Medeiros, L. O. Medeiros, *J. Anat.* **121**, 291–301 (1976).
6. D. R. Burgess, *Embryol. Exp. Morph.* **34**, 723–740 (1975).
7. W. His, *Anatomie Menschlicher Embryonen* (Vogel, Leipzig, Germany, 1880).
8. D. E. Moulton, A. Goriely, *J. Mech. Phys. Solids* **59**, 525–537 (2011).
9. L. Bell, L. Williams, *Anat. Embryol.* **165**, 437–455 (1982).
10. M. Kurahashi et al., *Neurogastroenterol. Motil.* **20**, 521–531 (2008).
11. K. Fukuda, Y. Tanigawa, G. Fujii, S. Yasugi, S. Hirohashi, *Development* **125**, 3535–3542 (1998).
12. H. Benabdallah, D. Messaoudi, K. Gharzouli, *Pharmacol. Res.* **57**, 132–141 (2008).

13. N. Harada, Y. Chijiwa, T. Misawa, M. Yoshinaga, H. Nawata, *Life Sci.* **51**, 1381–1387 (1992).
14. M. L. Lovett, C. M. Cannizzaro, G. Vunjak-Novakovic, D. L. Kaplan, *Biomaterials* **29**, 4650–4657 (2008).
15. N. Bowden, S. Brittain, A. G. Evans, J. W. Hutchinson, G. W. Whitesides, *Nature* **393**, 146–149 (1998).
16. L. Mahadevan, S. Rica, *Science* **307**, 1740 (2005).
17. B. Audoly, A. Boudaoud, *J. Mech. Phys. Solids* **56**, 2444–2458 (2008).
18. E. Hannezo, J. Prost, J.-F. Joanny, *Phys. Rev. Lett.* **107**, 078104 (2011).
19. M. Ben Amar, F. Jia, *Proc. Natl. Acad. Sci. U.S.A.* **110**, 10525–10530 (2013).
20. R. Sbarbati, *J. Anat.* **135**, 477–499 (1982).
21. K. D. Walton et al., *Proc. Natl. Acad. Sci. U.S.A.* **109**, 15817–15822 (2012).
22. A. Sukegawa et al., *Development* **127**, 1971–1980 (2000).
23. M. Ramalho-Santos, D. A. Melton, A. P. McMahon, *Development* **127**, 2763–2772 (2000).
24. T. Tallinen, J. S. Biggins, L. Mahadevan, *Phys. Rev. Lett.* **110**, 024302 (2013).
25. M. Daga et al., *Int. J. Dev. Biol.* **34**, 205–218 (1990).
26. Z. Uni, A. Smirnov, D. Sklan, *Poult. Sci.* **82**, 320–327 (2003).
27. F. T. Bellware, T. W. Betz, *J. Embryol. Exp. Morphol.* **24**, 335–355 (1970).
28. T. Sato et al., *Nature* **459**, 262–265 (2009).
29. W. H. St. Clair, C. A. Stahlberg, J. W. Osborne, *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol.* **47**, 27–33 (1984).
30. T. Savin et al., *Nature* **476**, 57–62 (2011).

Acknowledgments: We thank M. Kirschner for providing *Xenopus* tadpoles and O. Pourquie for providing snake embryos. D.L.K. and Tufts University hold a series of patents that cover the processing of silk into material structures, including those used in the research reported here. T.T. acknowledges the Academy of Finland for support. Computations were run at CSC—IT Center for Science, Finland. C.J.T. acknowledges the support of a grant from NIH R01 HD047360. L.M. acknowledges the support of the MacArthur Foundation.

Supplementary Materials

www.sciencemag.org/content/342/6155/212/suppl/DC1

Materials and Methods

Supplementary Text

Figs. S1 to S11

Movies S1 to S3

8 April 2013; accepted 13 August 2013

Published online 29 August 2013;

10.1126/science.1238842

REPORTS

Evidence for Water in the Rocky Debris of a Disrupted Extrasolar Minor Planet

J. Farihi,^{1*} B. T. Gänsicke,² D. Koester³

The existence of water in extrasolar planetary systems is of great interest because it constrains the potential for habitable planets and life. We have identified a circumstellar disk that resulted from the destruction of a water-rich and rocky extrasolar minor planet. The parent body formed and evolved around a star somewhat more massive than the Sun, and the debris now closely orbits the white dwarf remnant of the star. The stellar atmosphere is polluted with metals accreted from the disk, including oxygen in excess of that expected for oxide minerals, indicating that the parent body was originally composed of 26% water by mass. This finding demonstrates that water-bearing planetesimals exist around A- and F-type stars that end their lives as white dwarfs.

The enormous recent progress in the discovery of exoplanetary systems provides a growing understanding of their frequency

and nature, but our knowledge is still limited in many respects. There is now observational evidence of rocky exoplanets (1, 2), and the mass

and radius (and hence density) of these planets can be calculated from transit depth and radial velocity amplitude; however, estimates of their bulk composition remain degenerate and model-dependent. Transit spectroscopy offers some information on giant exoplanet atmospheres (3), and planetesimal debris disks often reveal the signature of emitting dust and gas species (4), yet both techniques only scratch the surface of planets, asteroids, and comets. Interestingly, white dwarfs—the Earth-sized embers of stars like the Sun—offer a unique window onto terrestrial exoplanetary systems: These stellar remnants can distill entire

¹Institute of Astronomy, University of Cambridge, Cambridge CB3 0HA, UK. ²Department of Physics, University of Warwick, Coventry CV5 7AL, UK. ³Institut für Theoretische Physik und Astrophysik, University of Kiel, 24098 Kiel, Germany.

*Corresponding author. E-mail: jfarihi@ast.cam.ac.uk

planetesimals into their constituent elements, thus providing the bulk chemical composition for the building blocks of solid exoplanets.

Owing to high surface gravities, any atmospheric heavy elements sink rapidly as white dwarfs cool below 25,000 K (5), leaving behind only hydrogen and helium in their outermost layers—a prediction that is corroborated by observation (6). Those white dwarfs with rocky planetary system remnants can become contaminated by the accretion of small, but spectroscopically detectable, amounts of metals (7). Heavy element absorption lines in cool white dwarfs are a telltale of external pollution, often implying either ongoing mass accretion rates above 10^8 g s^{-1} (8) or large asteroid-sized masses of metals within the convection zone of the star (9).

In recent years, metal-rich dust (10, 11) and gas (12) disks, likely produced by the tidal disruption of a large asteroid (13), have been observed to be closely orbiting 30 cool white dwarfs [e.g., (14–19)] and provide a ready explanation for the metal absorption features seen in their atmospheres (20). The circumstellar material being gradually accreted by the white dwarf can be directly observed in the stellar photosphere to reveal its elemental abundances (21). These planetary system remnants offer empirical insight into the assembly and chemistry of terrestrial exoplanets that is unavailable for any exoplanet orbiting a main-sequence star.

Until now, no white dwarf has shown reliable evidence for the accretion of water-rich, rocky planetary material. Unambiguous signatures of icy asteroids at white dwarfs should include (i) atmospheric metal pollution rich in refractory elements; (ii) trace oxygen in excess of that expected for metal oxides; (iii) circumstellar debris from which these elements are accreted; and, where applicable, (iv) trace hydrogen (in a helium-dominated atmosphere) sufficient to account for the excess oxygen as H_2O . The presence of a circumstellar disk signals that accretion is ongoing, identifies the source material, and enables a confident quantitative assessment of the accreted elemental abundances, which in

turn allows a calculation of the water fraction of the disrupted parent body.

The metal-enriched white dwarfs GD 362 and GD 16 both have circumstellar disks and relatively large trace hydrogen abundances in helium-dominated atmospheres (22), but as yet no assessment of photospheric oxygen is available (21, 23). These two stars have effective temperatures below 12,000 K, and their trace hydrogen could potentially be the result of helium dredge-up in a previously hydrogen-rich atmosphere (24). The warmer, metal-lined white dwarfs GD 61 and GD 378 have photospheric oxygen (25), but the accretion history of GD 378 is unconstrained (i.e., it does not have a detectable disk), and without this information, the atmospheric oxygen could be consistent with that contained in dry minerals common in the inner solar system (26). In the case of GD 61, elemental abundance uncertainties have previously prevented a formally significant detection of oxygen excess (27).

We used the Cosmic Origins Spectrograph (COS) onboard the Hubble Space Telescope to obtain ultraviolet spectroscopy of the white dwarf GD 61, and, together with supporting ground-based observations, we derived detections or limits for all the major rock-forming elements (O, Mg, Al, Si, Ca, Fe). These data permit a confident evaluation of the total oxygen fraction present in common silicates within the parent body of the infalling material, and we identified excess oxygen attributable to H_2O as follows. (i) The observed carbon deficiency indicates that this element has no impact on the total oxygen budget, even if every atom is delivered as CO_2 . (ii) The elements Mg, Al, Si, and Ca are as-

sumed to be carried as MgO , Al_2O_3 , SiO_2 , and CaO at the measured or upper-limit abundance. (iii) The remaining oxygen exceeds that which can be bound in FeO , and the debris is interpreted to be water-rich. By this reasoning, we found oxygen in excess of that expected for anhydrous minerals in the material at an H_2O mass fraction of 0.26 (Table 1 and Fig. 1).

Because we have assumed the maximum allowed FeO , and because some fraction of metallic iron is possible, the inferred water fraction of the debris is actually bound between 0.26 and 0.28. Although this makes little difference in the case of GD 61, where the parent body material appears distinctly mantle-like (27), there are at least two cases where metallic iron is a major (and even dominant) mass carrier within the parent bodies of circumstellar debris observed at white dwarfs (28). Overall, these data strongly suggest that the material observed in and around polluted white dwarfs had an origin in relatively massive and differentiated planetary bodies.

We have assumed a steady state between accretion and diffusion in GD 61. However, a typical metal sinking time scale for this star is 10^5 years, and thus the infalling disk material could potentially be in an early phase of accretion where material accumulates in the outer layers, prior to appreciable sinking (27). In this early-phase scenario, the oxygen excess and water fraction would increase relative to those derived from the steady-state assumption, and hence we confidently conclude that the debris around GD 61 originated in a water-rich parent body. Although the lifetimes of disks at white dwarfs are not robustly constrained, the best estimates imply

Table 1. Oxide and water mass fractions in the planetary debris at GD 61. We adopt the steady-state values, which assume accretion-diffusion equilibrium.

Oxygen carrier	Steady state	Early phase
CO_2	<0.002	<0.002
MgO	0.17	0.18
Al_2O_3	<0.02	<0.02
SiO_2	0.32	0.27
CaO	0.02	0.01
FeO^*	0.05	0.02
Excess	0.42	0.50
H_2O in debris	0.26	0.33

*All iron is assumed to be contained in FeO ; some metallic Fe will modestly increase the excess oxygen.

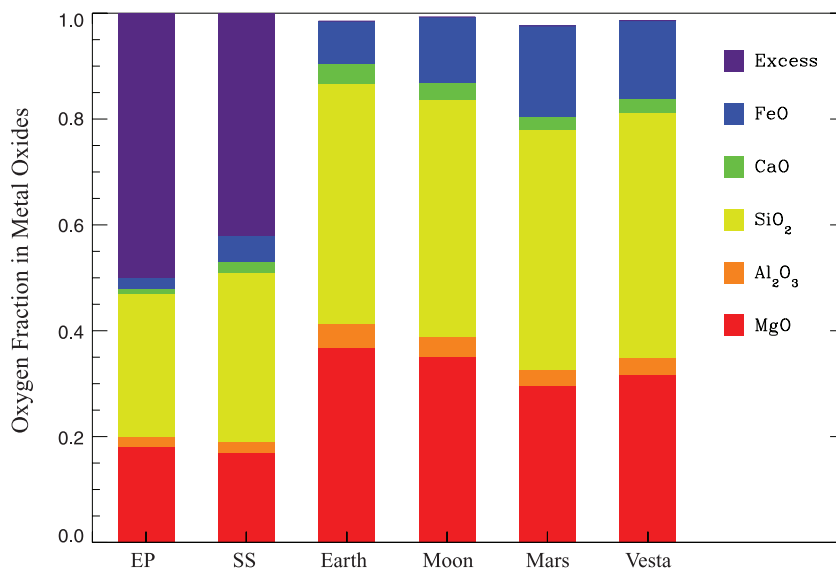


Fig. 1. Oxygen budget in GD 61 and terrestrial bodies. The first two columns are the early phase (EP) and steady-state (SS) fractions of oxygen carried by all the major rock-forming elements in GD 61, assuming that all iron is carried as FeO . Additional columns show the oxide compositions of the bulk silicate (crust plus mantle) Earth, Moon, Mars, and Vesta (35). Their totals do not reach 1.0 because trace oxides have been omitted. The overall chemistry of GD 61 is consistent with a body composed almost entirely of silicates, and thus appears relatively mantle-like but with substantial water. In contrast, Earth is relatively water-poor and contains approximately 0.023% H_2O ($1.4 \times 10^{24} \text{ g}$).

that the chance of catching GD 61 in an early phase is less than 1% (17, 29–31).

The helium-rich nature of GD 61 permits an assessment of its trace hydrogen content and total asteroid mass for a single parent body. The total metal mass within the stellar convection zone is 1.3×10^{21} g, roughly equivalent to that of an asteroid 90 km in diameter. However, because metals continuously sink, it is expected that the destroyed parent body was substantially more massive, unless the star is being observed shortly after the disruption event. In contrast, hydrogen floats and accumulates, and thus places an upper limit on the total mass of accreted water-rich debris. If all the trace hydrogen were delivered as H_2O from a single planetesimal, the total accreted water mass would be 5.2×10^{22} g, and a 26% H_2O mass fraction would imply a parent body mass of 2×10^{23} g, which is similar to that of the main-belt asteroid 4 Vesta (32).

These data imply that water in planetesimals can survive post-main sequence evolution. One possibility is that solid or liquid water is retained beneath the surface of a sufficiently large (diameter >100 km) parent body (26), and is thus protected from heating and vaporization by the outermost layers. Upon shattering during a close approach with a white dwarf, any exposed water ice (and volatiles) should rapidly sublimate but will eventually fall onto the star; the feeble luminosities of white dwarfs are incapable of removing even light gases by radiation pressure (31). Another possibility is that a substantial mass of water is contained in hydrated minerals (e.g., phyllosilicates), as observed in main-belt asteroids via spectroscopy and inferred from the analysis of meteorites (33). In this case, the H_2O equivalent is not removed until much higher temperatures are attained, and such water-bearing asteroids may remain essentially unaffected by the giant phases of the host star.

The white dwarf GD 61 contains the unmistakable signature of a rocky minor planet analogous to the asteroid 1 Ceres in water content (34) and probably analogous to Vesta in mass. The absence of detectable carbon indicates that the parent body of the circumstellar debris was not an icy planetesimal analogous to comets, but was instead similar in overall composition to asteroids in the outer main belt. This exoplanetary system originated around an early A-type star that formed large planetesimals similar to those in the inner solar system that were the building blocks for Earth and other terrestrial planets.

References and Notes

- N. M. Batalha *et al.*, *Astrophys. J.* **729**, 27 (2011).
- F. Fressin *et al.*, *Nature* **482**, 195–198 (2012).
- D. K. Sing *et al.*, *Mon. Not. R. Astron. Soc.* **416**, 1443–1455 (2011).
- C. M. Lisse *et al.*, *Astrophys. J.* **747**, 93 (2012).
- D. Koester, *Astron. Astrophys.* **498**, 517–525 (2009).
- B. Zuckerman, D. Koester, I. N. Reid, M. Hünsch, *Astrophys. J.* **596**, 477–495 (2003).
- Astronomers use the term “metal” when referring to elements heavier than helium.

- D. Koester, D. Wilken, *Astron. Astrophys.* **453**, 1051–1057 (2006).
- J. Farihi, M. A. Barstow, S. Redfield, P. Dufour, N. C. Hambly, *Mon. Not. R. Astron. Soc.* **404**, 2123 (2010).
- M. Jura, J. Farihi, B. Zuckerman, *Astron. J.* **137**, 3191–3197 (2009).
- W. T. Reach *et al.*, *Astrophys. J.* **635**, L161–L164 (2005).
- B. T. Gänsicke, T. R. Marsh, J. Southworth, A. Rebassa-Mansergas, *Science* **314**, 1908–1910 (2006).
- J. H. Debes, K. J. Walsh, C. Stark, *Astrophys. J.* **747**, 148 (2012).
- J. Farihi *et al.*, *Mon. Not. R. Astron. Soc.* **421**, 1635–1643 (2012).
- J. Farihi, M. Jura, J. E. Lee, B. Zuckerman, *Astrophys. J.* **714**, 1386–1397 (2010).
- S. Xu, M. Jura, *Astrophys. J.* **745**, 88 (2012).
- J. Girven *et al.*, *Astrophys. J.* **749**, 154 (2012).
- J. Farihi, M. Jura, B. Zuckerman, *Astrophys. J.* **694**, 805–819 (2009).
- M. Jura, J. Farihi, B. Zuckerman, *Astrophys. J.* **663**, 1285–1290 (2007).
- M. Jura, *Astrophys. J.* **584**, L91–L94 (2003).
- B. Zuckerman, D. Koester, C. Melis, B. M. S. Hansen, M. Jura, *Astrophys. J.* **671**, 872–877 (2007).
- M. Jura, M. Muno, J. Farihi, B. Zuckerman, *Astrophys. J.* **699**, 1473–1479 (2009).
- D. Koester, R. Napiwotzki, B. Voss, D. Homeier, D. Reimers, *Astron. Astrophys.* **439**, 317–321 (2005).
- P. E. Tremblay, P. Bergeron, *Astrophys. J.* **672**, 1144–1152 (2008).
- S. Desharnais, F. Wesemael, P. Chayer, J. W. Kruk, R. A. Saffer, *Astrophys. J.* **672**, 540–552 (2008).
- M. Jura, S. Xu, *Astron. J.* **140**, 1129–1136 (2010).
- J. Farihi *et al.*, *Astrophys. J.* **728**, L8 (2011).
- B. T. Gänsicke *et al.*, *Mon. Not. R. Astron. Soc.* **424**, 333–347 (2012).
- B. Klein, M. Jura, D. Koester, B. Zuckerman, C. Melis, *Astrophys. J.* **709**, 950–962 (2010).
- M. Jura, *Astron. J.* **135**, 1785–1792 (2008).
- J. Farihi, B. Zuckerman, E. E. Becklin, *Astrophys. J.* **674**, 431–446 (2008).
- C. T. Russell *et al.*, *Science* **336**, 684–686 (2012).
- A. S. Rivkin, E. S. Howell, F. Vilas, L. A. Lebofsky, in *Asteroids III*, W. F. Bottke Jr., A. Cellino, P. Paolicchi, R. P. Binzel, Eds. (Univ. of Arizona Press, Tucson, AZ, 2002), pp. 235–253.
- P. C. Thomas *et al.*, *Nature* **437**, 224–226 (2005).
- C. Visscher, B. Fegley Jr., *Astrophys. J.* **767**, L12 (2013).

Acknowledgments: This work is based on observations made with the Hubble Space Telescope, which is operated by the Association of Universities for Research in Astronomy under NASA contract NAS 5-26555. These observations are associated with program programs 12169 and 12474. Some of the data presented herein were obtained at the W. M. Keck Observatory, which is operated as a scientific partnership among the California Institute of Technology, the University of California, and NASA. The Observatory was made possible by the generous financial support of the W. M. Keck Foundation. J.F. acknowledges support from the UK Science and Technology Facilities Council in the form of an Ernest Rutherford Fellowship (ST/J003344/1). The research leading to these results has received funding from the European Research Council under the European Union's Seventh Framework Programme (FP/2007-2013)/ERC Grant Agreement no. 267697 (WDTracer). B.T.G. was supported in part by the UK Science and Technology Facilities Council (ST/I001719/1). Keck telescope time for program 2011B-0554 was granted by NOAO through the Telescope System Instrumentation Program, funded by NSF.

Supplementary Materials

www.sciencemag.org/content/342/6155/218/suppl/DC1
Materials and Methods
Fig. S1
Tables S1 and S2
References (36, 37)

22 April 2013; accepted 15 August 2013
10.1126/science.1239447

Femtosecond Visualization of Lattice Dynamics in Shock-Compressed Matter

D. Milathianaki,^{1*} S. Boutet,¹ G. J. Williams,¹ A. Higginbotham,² D. Ratner,¹ A. E. Gleason,³ M. Messerschmidt,¹ M. M. Seibert,^{1,4} D. C. Swift,⁵ P. Hering,¹ J. Robinson,¹ W. E. White,¹ J. S. Wark²

The ultrafast evolution of microstructure is key to understanding high-pressure and strain-rate phenomena. However, the visualization of lattice dynamics at scales commensurate with those of atomistic simulations has been challenging. Here, we report femtosecond x-ray diffraction measurements unveiling the response of copper to laser shock-compression at peak normal elastic stresses of ~73 gigapascals (GPa) and strain rates of 10^9 per second. We capture the evolution of the lattice from a one-dimensional (1D) elastic to a 3D plastically relaxed state within a few tens of picoseconds, after reaching shear stresses of 18 GPa. Our in situ high-precision measurement of material strength at spatial (<1 micrometer) and temporal (<50 picoseconds) scales provides a direct comparison with multimillion-atom molecular dynamics simulations.

The distinct properties of materials at high-pressure and/or strain-rate conditions lead to a broad range of phenomena in fields such as high-energy-density physics (1), Earth and planetary sciences (2, 3), aerospace engineering (4), and materials science (5, 6). For the latter, a predictive understanding and control of mechanical properties, enabled by the di-

rect comparison of experiments with large-scale atomistic simulations, is the ultimate goal. Whereas the bulk material behavior can be inferred by macroscopic measurements (7, 8), key information on the mechanical properties requires knowledge of the physics embedded at the lattice level. Such knowledge has traditionally been obtained via nanosecond-resolution x-ray

diffraction measurements (9–14) from dynamically compressed samples that are tens of micrometers in thickness.

In recent years, molecular dynamics (MD) simulations (15–18) on massively parallel computers have elucidated the orientation-dependent response of single-crystal samples to shock and ramp compression (19, 20). Because of computational complexities, such simulations have interrogated spatial scales of $<1\ \mu\text{m}$ and temporal scales of $<1\ \text{ns}$. Specifically, MD simulations have predicted that for picosecond

compression time scales, the response of a face-centered cubic crystal, such as Cu, should initially be elastic up to very high strains (12 to 20%), depending on crystallographic orientation and strain rate (15, 18). Similar behavior has been suggested by dislocation dynamics (DD) simulations (21). However, these results have remained largely unverified, either because in situ strain measurements have been indirect (22, 23) or because they had limited temporal and/or angular resolution (9, 11, 12). We present high-precision x-ray diffraction measurements of the lattice evolution in laser-shocked polycrystalline Cu (24). A sequence of femtosecond snapshots at pump-probe intervals of 10 ps (corresponding to an incremental shock propagation distance of $\sim 55\ \text{nm}$) produced a lattice-level movie of the strain state within the material. As the x-ray pulse length was less than even the shortest phonon period, diffraction captured strain pro-

files in the absence of temporal smearing. Furthermore, the diffraction geometry was designed to differentiate between a purely elastic response and plastic relaxation resulting from the generation and motion of dislocations. The sample thickness ($1\ \mu\text{m}$), stress rise time ($<80\ \text{ps}$), and propagation time ($\sim 180\ \text{ps}$) were directly comparable with the spatial and temporal dimensions of MD simulations.

The experiment was performed at the Coherent X-ray Imaging Instrument (25) of the Linac Coherent Light Source (LCLS). An ablation-driven compression front was launched parallel to the sample normal and across a Gaussian $260\text{-}\mu\text{m}$ ($1/e^2$ diameter, where e is equal to 2.718281828) focus using the 800 nm, $\leq 20\ \text{mJ}$, $\sim 170\ \text{ps}$ (full width at half-maximum) output of a Ti:sapphire laser system (Fig. 1). The texture of the polycrystalline Cu samples ($\sim 400\text{-nm}$ average grain size) was such that crystallites were

¹SLAC National Accelerator Laboratory, Menlo Park, CA 94025, USA. ²Department of Physics, Clarendon Laboratory, University of Oxford, Oxford OX1 3PU, UK. ³Department of Geological and Environmental Sciences, Stanford University, Stanford, CA 94305, USA. ⁴Department of Cell and Molecular Biology, Uppsala University, 751 24 Uppsala, Sweden. ⁵Lawrence Livermore National Laboratory, Livermore, CA 94551, USA.

*Corresponding author. E-mail: despina@slac.stanford.edu

Fig. 1. Experimental configuration of the pump (optical laser) and lattice probe (LCLS) scheme. The lattice response of the shock-compressed $1\text{-}\mu\text{m}$ polycrystalline Cu films deposited on $85\text{-}\mu\text{m}$ $<100>$ Si substrates was captured in a Debye-Scherrer geometry by a series of 48-fs snapshots. A preferential orientation of the Cu crystallites constrained the axis of the applied stress along the $[111]$ direction. FEL, free electron laser; θ_B , Bragg angle.

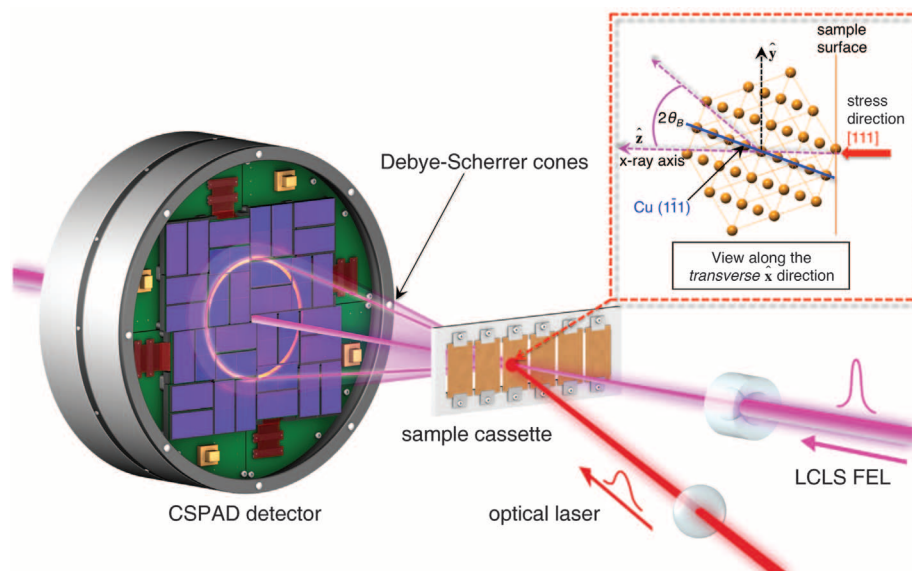
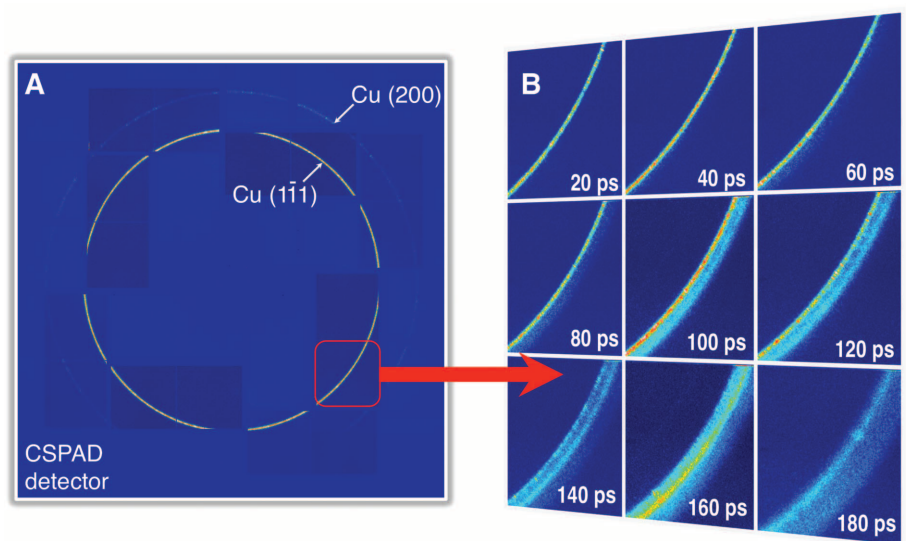


Fig. 2. Raw data illustrating the Debye-Scherrer cone projections captured on the CSPAD detector. (A) Projections from the Cu $(1\bar{1}1)$ and Cu (200) planes are displayed. The intensity of the Cu $(1\bar{1}1)$ plane is higher than expected from the calculated structure factors for a powder specimen because of texture. (B) A section of the diffraction ring is magnified, and its evolution is shown at 20-ps intervals. Movies S1 and S2 demonstrate the angular shift in the x-ray signal as a function of the lattice strain state, recorded at 10-ps intervals.



preferentially shocked along the $\langle 111 \rangle$ direction. Quasi-monochromatic ($\Delta E/E = 0.2$ to 0.5% , where E is energy and ΔE is the change in energy) 8.8-keV x-ray pulses with 48-fs duration and an average of $\sim 10^{12}$ photons per pulse were incident over a 30-by-30- μm^2 spot. Diffraction rings were recorded from the $(1\bar{1}1)$ reflections on an in-vacuum, 2.3-megapixel array detector [the Cornell Stanford Pixel Array Detector (CSPAD)] (26) in a Debye-Scherrer geometry (27). Pump-probe delay scans with incremental 10-ps intervals allowed us to obtain a time series of x-ray diffraction patterns from the shock-compressed lattice (28).

A full diffraction ring from the ambient sample is shown in Fig. 2A, where the uniformity in signal intensity indicates rotational symmetry of the crystallites around the x-ray and shock axis. As the sample is uniaxially compressed, the strain profile across the sample depth results in an angular distribution of the diffracted x-rays that depends on the instantaneous elastic strains both parallel and perpendicular to the shock propagation direction (28) (Fig. 2B and movies S1 and

S2). Our Debye-Scherrer geometry allows a clear demarcation of elastic and hydrostatic responses. Specifically, the low ambient Bragg angle (19.8°) leads to a substantial difference in the angular shift of the x-rays for a given elastic strain along the shock propagation direction; if the sample response is purely elastic (i.e., ϵ_n^e , the elastic strain along the shock direction, is finite, but $\epsilon_t^e = -\epsilon_t^p = 0$, where ϵ_t^e is the transverse elastic strain, and ϵ_t^p the transverse plastic strain) the angular shift is almost nine times less than that of a nearly hydrostatic response (where $\epsilon_n^e = \epsilon_t^e = -\epsilon_t^p$) (28).

Azimuthal integration of the diffracted intensities as a function of x-ray scattering angle 2θ and for different delay times (Fig. 3A) shows (i) a reduction in intensity of the ambient $(1\bar{1}1)$ diffraction peak at $2\theta_0 = 39.6^\circ$; (ii) the subsequent emergence of a diffraction peak at $2\theta = 40.4^\circ$, an angle that remains constant with laser irradiance (fig. S1); and (iii) a broad feature extending to scattering angle $2\theta \sim 43^\circ$ at later delays. An understanding of these features in the time-dependent diffraction profiles can be gained via

comparison with simulated strain and diffraction profiles (Fig. 3, B to D) calculated from a hydrocode incorporating a simple plasticity model (28).

To distinguish the diffraction features representative of the shock-induced strain state in the lattice, we divide the range of scattering angles into regions I, II, and III (Fig. 3A). At any instant in time, the diffraction patterns are a superposition of the depth-dependent strain states along the entire sample thickness, as the x-ray probe depth at 8.8 keV in Cu is $\gg 1 \mu\text{m}$. The prominent feature in region I is the diffraction peak from the unstrained $(1\bar{1}1)$ lattice plane at $2\theta_0 = 39.6^\circ$. As the compression front traverses the sample, the ambient diffraction peak intensity decreases because of the reduction in thickness of unstrained material. Within the first 40 ps of shock propagation, the lattice exhibits a considerable elastic response that is evident in region II. Such response can be attributed to an initial homogeneous nucleation regime, as predicted by MD and DD simulations (15, 21), with duration depending

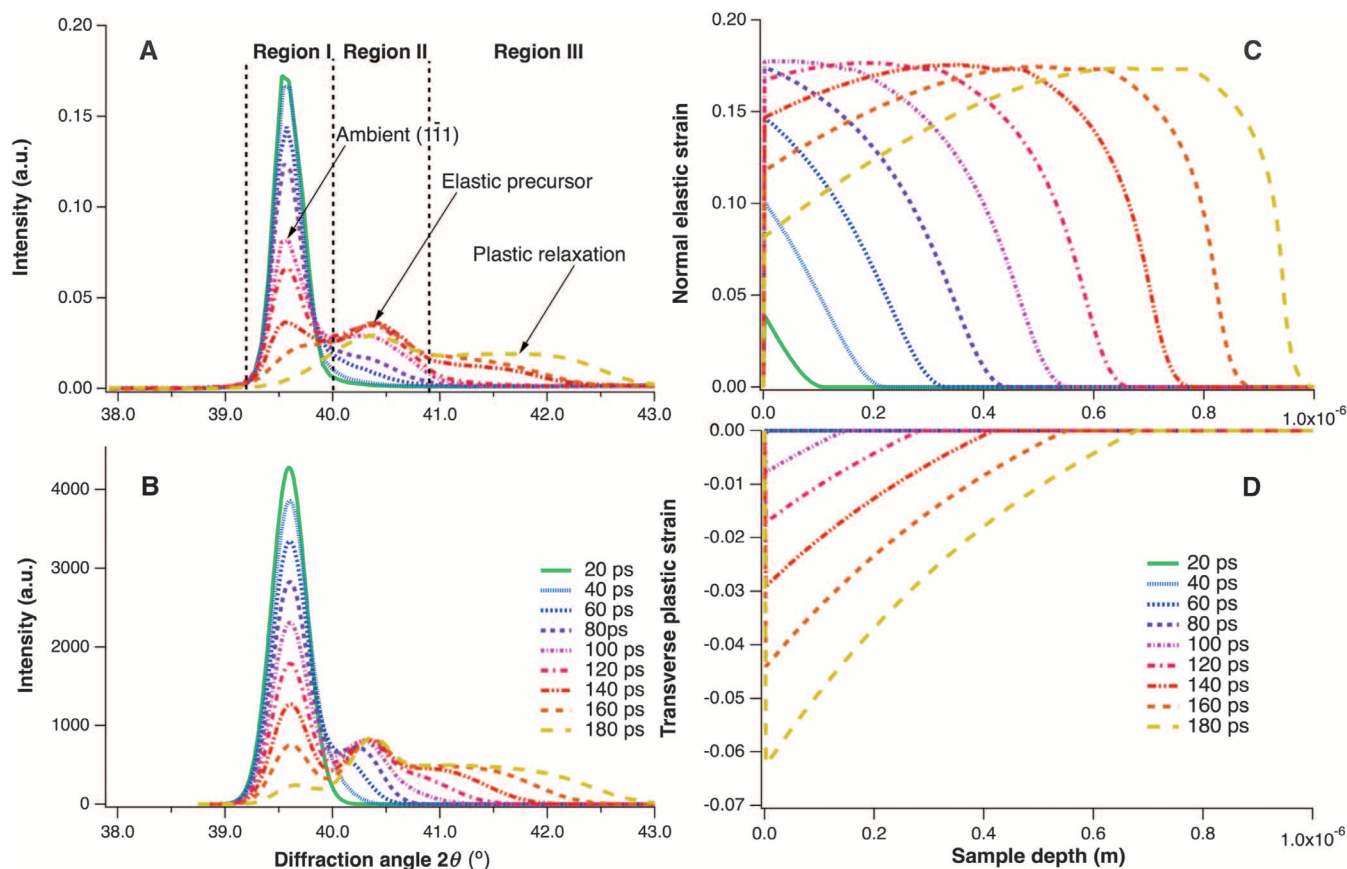


Fig. 3. Elastic and plastic strain profiles in shocked Cu and corresponding diffraction signal, as calculated from the captured x-ray data. (A) Experimental diffraction data resulting from the azimuthal integration around 2π of the Cu $(1\bar{1}1)$ x-ray signal. The diffraction profiles are divided into three regions to illustrate the characteristic lattice response: region I, the unstrained lattice; region II, the elastically compressed lattice; and region III, the lattice exhibiting three-dimensional relaxation. a.u., arbitrary units. **(B)** Simulated

diffraction data resulting from the calculated strain profiles, showing good agreement with experiment. Discrepancies in the signal amplitude are mainly due to artifacts introduced in the azimuthal integration of the diffraction peaks by the area detector tiling, as well as the $\Delta t = 0$ synchronization uncertainty (~ 20 ps) in the time of arrival of the optical and x-ray pulse. **(C)** The modeled time-dependent normal elastic strain and **(D)** transverse plastic strain profiles versus sample depth.

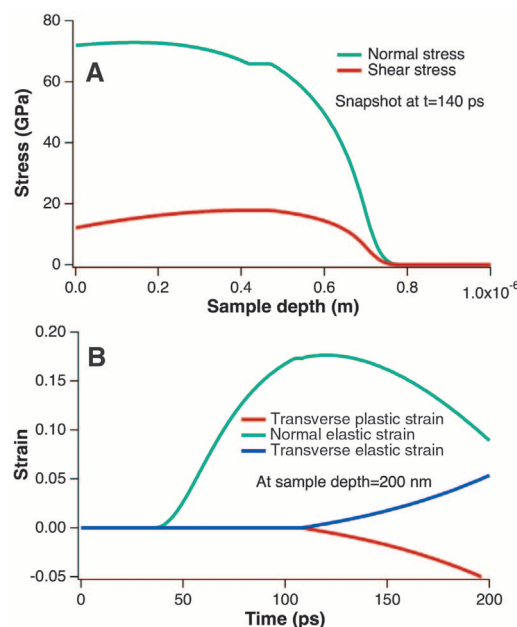
on the rise time of the compression front. A large elastic strain ϵ_n^e of 18% is induced along the shock propagation axis, as manifested by an angular shift of the ambient diffraction peak to $2\theta = 40.4^\circ$. Note that in this experimental geometry, such normal elastic strain value corresponds to a relatively low angular shift (0.8°) of the ambient diffraction peak as $\Delta\theta \sim \tan\theta_0 \sin^2\theta_0 \times \epsilon_n^e$ in the absence of plastic flow. Other notable features of this elastic diffraction peak are (i) amplitude that increases with time, as the thickness of the elastically compressed region also increases, and (ii) narrow angular distribution because of the low elastic strain gradient present (Fig. 3C). Our calculations, based on the experimental shock parameters and captured diffraction profiles, indicate that this purely elastic response persists up to a peak normal stress of ~ 73 GPa and shear stress of 18 GPa (Fig. 4A). The latter, representing the yield stress of the material, is in excellent agreement with MD simulations in single crystal Cu (18) at a strain rate of (10^9 s $^{-1}$) and for uniaxial compression along the [111] direction, thus confirming the considerably higher yield stress values predicted by simulations compared with those extracted from nanosecond shock experiments on samples of $\gg 1$ - μ m thickness (12).

Plastic deformation is induced when the normal elastic stress exceeds the material elastic limit, in this case ~ 73 GPa, causing multiplication and motion of existing dislocations. Region III encompasses diffraction from regions of the lattice evolving from a purely elastic to a nearly hydrostatic state after compression to the elastic limit; here, whereas the magnitude of ϵ_n^e decreases with time, the magnitude of $\epsilon_t^e = -\epsilon_t^p$ increases (Fig. 4B). Despite ϵ_n^e being much lower than for the elastic regime of region II, the change in the scattering angle is

considerably larger, as in the hydrostatic limit $\Delta\theta \sim -\tan\theta_0 \times \epsilon_t^p$. Scattering extends out to $2\theta \sim 43^\circ$, consistent with a plastic strain state of $\epsilon_t^p = -6.2\%$. The plastic strain rate $\dot{\epsilon}_t^p$ can be estimated at $\sim 10^9$ s $^{-1}$, considering that $\epsilon_t^p = -6.2\%$ is reached over a time period of ~ 60 ps. Taking a magnitude $b = 2.6$ Å for the Burgers vector, we deduce a dislocation density-velocity product of 4.0×10^{18} m $^{-1}$ s $^{-1}$ via Orowan's equation (29) $\dot{\epsilon}_t^p = A\rho_m b v$, where constant A is of order unity, ρ_m is the mobile dislocation density, and v is the average dislocation velocity. We note that MD (15) simulations predict average dislocation velocities of ≤ 1300 m s $^{-1}$ at similar strain rates, implying a minimum dislocation density in our experiment of 3.0×10^{15} m $^{-2}$. This value, although lower than the MD simulations (10^{17} m $^{-2}$) (15), is in close agreement with that calculated in DD simulations (10^{16} m $^{-2}$) (21). This discrepancy could be a consequence of the methods used to extract dislocation densities or of dislocation tangling and locking, which result in the total number of dislocations produced in MD to be artificially higher than the number of mobile dislocations responsible for plastic flow. We note that, in the future, small-angle scattering techniques exploiting the high spectral brightness of LCLS could provide direct information on the transient dislocation density itself.

Our results highlight the necessity for experiments designed with length scales equal to these of atomistic simulations; such fundamental measurements of the material response could be used to evaluate and improve time-dependent deformation models, thus extending our knowledge on the ultrafast elastic-plastic behavior of different crystal structures. Beyond simple crystal structures, the mechanical properties of complex engineered materials (30) could also be studied and optimized.

Fig. 4. Snapshot of the normal and shear stress in the sample and strain history. (A) Calculated normal and shear stress from the experimentally determined angular shift in the Cu (111) reflection at $t = 140$ ps. **(B)** The elastic and plastic strain history at a sample depth of 200 nm. Note that plastic relaxation initiates when the normal elastic strain reaches a peak value of $\sim 18\%$.



References and Notes

- W. J. Nellis, *Rep. Prog. Phys.* **69**, 1479–1580 (2006).
- L. Dubrovinsky *et al.*, *Science* **316**, 1880–1883 (2007).
- R. S. McWilliams *et al.*, *Science* **338**, 1330–1333 (2012).
- K. Thoma, F. Schafer, S. Hiermaier, E. Schneider, *Adv. Space Res.* **34**, 1063–1075 (2004).
- E. M. Bringa *et al.*, *Science* **309**, 1838–1841 (2005).
- M. W. Chen, J. W. McCauley, D. P. Dandekar, N. K. Bourne, *Nat. Mater.* **5**, 614–618 (2006).
- C. A. Bolme, S. D. McGrane, D. S. Moore, D. J. Funk, *J. Appl. Phys.* **102**, 033513 (2007).
- P. M. Celliers *et al.*, *Rev. Sci. Instrum.* **81**, 035101 (2010).
- A. Loveridge-Smith *et al.*, *Phys. Rev. Lett.* **86**, 2349–2352 (2001).
- D. H. Kalantar *et al.*, *Phys. Rev. Lett.* **95**, 075502 (2005).
- B. J. Jensen, Y. M. Gupta, *J. Appl. Phys.* **104**, 013510 (2008).
- W. J. Murphy *et al.*, *J. Phys. Condens. Matter* **22**, 065404 (2010).
- D. Milathianaki *et al.*, *Phys. Rev. B* **86**, 014101 (2012).
- M. J. Suggit *et al.*, *Nat. Commun.* **3**, 1224 (2012).
- E. M. Bringa *et al.*, *Nat. Mater.* **5**, 805–809 (2006).
- K. Kadau *et al.*, *Phys. Rev. Lett.* **98**, 135701 (2007).
- A. Higginbotham *et al.*, *Phys. Rev. B* **85**, 024112 (2012).
- V. Dupont, T. C. Germann, *Phys. Rev. B* **86**, 134111 (2012).
- D. C. Swift, T. E. Tierney IV, R. A. Kopp, J. T. Gammel, *Phys. Rev. E* **69**, 036406 (2004).
- D. C. Swift *et al.*, *Phys. Rev. E* **78**, 066115 (2008).
- M. A. Shehadeh, E. M. Bringa, H. M. Zbib, J. M. McNaney, B. A. Remington, *Appl. Phys. Lett.* **89**, 171918 (2006).
- J. M. Winey, B. M. LaLone, P. B. Trivedi, Y. M. Gupta, *J. Appl. Phys.* **106**, 073508 (2009).
- J. C. Crowhurst, M. R. Armstrong, K. B. Knight, J. M. Zaugg, E. M. Behymer, *Phys. Rev. Lett.* **107**, 144302 (2011).
- P. Emma *et al.*, *Nat. Photonics* **4**, 641–647 (2010).
- S. Boutet, G. J. Williams, *New J. Phys.* **12**, 035024 (2010).
- H. T. Philipp, M. Hromalik, M. Tate, L. Koerner, S. M. Gruner, *Nucl. Instrum. Methods Phys. Res. A* **649**, 67–69 (2011).
- B. D. Cullity, S. R. Stock, *Elements of X-ray Diffraction* (Prentice Hall, Upper Saddle River, NJ, ed. 3, 2001).
- See supplementary materials on Science Online.
- E. Orowan, *Nature* **149**, 643–644 (1942).
- M. F. Chisholm, S. Kumar, P. Hazzledine, *Science* **307**, 701–703 (2005).

Acknowledgments: We thank M. Bionta, A. Fry, S. Edstrom, J. Koglin, S. Guillet, I. Ofte, and G. Stewart for assisting with our experimental requirements, data processing, and illustrations. Raw x-ray data are available upon request. This work was funded as part of the in-house research effort of LCLS, a National User Facility operated by Stanford University on behalf of the U.S. Department of Energy, Office of Basic Energy Sciences. A.H. acknowledges support from the UK Atomic Weapons Establishment and J.S.W. from the UK Engineering and Physical Sciences Research Council under grant EP/J017256/1.

Supplementary Materials

www.sciencemag.org/content/342/6155/220/suppl/DC1

Materials and Methods

Supplementary Text

Fig. S1

References (31–35)

Movies S1 and S2

24 April 2013; accepted 4 September 2013
10.1126/science.1239566

Imaging Atomic Rearrangements in Two-Dimensional Silica Glass: Watching Silica's Dance

Pinshane Y. Huang,¹ Simon Kurasch,^{2*} Jonathan S. Alden,^{1*} Ashivni Shekhawat,³ Alexander A. Alemi,³ Paul L. McEuen,^{3,4} James P. Sethna,³ Ute Kaiser,² David A. Muller^{1,4†}

Structural rearrangements control a wide range of behavior in amorphous materials, and visualizing these atomic-scale rearrangements is critical for developing and refining models for how glasses bend, break, and melt. It is difficult, however, to directly image atomic motion in disordered solids. We demonstrate that using aberration-corrected transmission electron microscopy, we can excite and image atomic rearrangements in a two-dimensional silica glass—revealing a complex dance of elastic and plastic deformations, phase transitions, and their interplay. We identified the strain associated with individual ring rearrangements, observed the role of vacancies in shear deformation, and quantified fluctuations at a glass/liquid interface. These examples illustrate the wide-ranging and fundamental materials physics that can now be studied at atomic-resolution via transmission electron microscopy of two-dimensional glasses.

Structural rearrangements play a key role in controlling the basic properties of glassy and disordered solids. Yet, studies of phenomena such as plastic deformation and phase change have been limited by the difficulty of tracking individual atoms in amorphous materials. As a result, many of the landmark studies on the atomic-scale underpinnings of these phenomena have been conducted by computer simulations (1–5) or with pseudo-atomic systems, such as micrometer-scale colloidal particles (6–8) or bubble-rafts (9), as stand-ins for atoms. To verify these findings, it is critical to develop experimental methods that can directly image the rearrangements of atoms in glasses. We demonstrate an approach to address this void: applying aberration-corrected high-resolution transmission electron microscopy (TEM) to image and restructure a two-dimensional (2D) silica glass (10, 11). We first investigated the basic building blocks of plastic deformation by characterizing the strain around an isolated ring rearrangement. By comparing our experimental data with atomistic simulations, we show that, whereas the glass's structural disorder strongly affects its long-range elastic behavior, its short-range strain field resembles that of a crystal. Next, we investigated how multiple rearrangements interact to produce shear. We observed distinct, localized zones whose differing motions each contributed to the larger-scale deformation. Finally, we analyzed rearrangements at a sharp, but fluctuating, glass/liquid interface.

Two-dimensional silica is a layered polymorph of SiO₂. Its crystalline phase is a honeycomb lattice with an in-plane lattice constant $a = 5.4$ Å. Out-of-plane, it consists of two registered lay-

ers of SiO₄ tetrahedra (10, 12). More interesting is the amorphous phase, shown in Fig. 1A and fig. S1. Amorphous 2D silica has an out-of-plane structure similar to that of its crystalline phase, but in-plane, it resembles the 2D continuous random network predicted by Zachariasen's model (13). Unlike typical 3D glasses, this 2D glass can be imaged at atomic resolution (10, 11)—generating considerable recent interest (14–16). Both phases can be synthesized in a chemical vapor deposition (CVD) furnace (10, 17) or grown by molecular beam epitaxy on metal substrates (11, 12). For this work, we used CVD-grown silica supported on a graphene substrate (17) from the same batch of samples as discussed in (10). The graphene serves as a mostly transparent, chemically inert imaging substrate (18, 19).

To image and induce atomic motion in the 2D silica, we used low-voltage, aberration-corrected

high-resolution TEM at 80 kV. Similar techniques have been used to visualize structural transformations such as molecular motions (20) and rearrangements in 2D crystals such as graphene (21–26). The electron beam produces broad-beam illumination (~ 100 nm in diameter) with typical dose rates of $\sim 1.4 \times 10^6$ electrons/nm² s and frame rates of ~ 1 to 2 s. With low probabilities, electrons can transfer sufficient local energy to eject atoms or break bonds through elastic or inelastic scattering (27–30). These processes result in mobile (mainly oxygen) vacancies, which produce strain and enable plastic deformation, flow, and even local melting well below the glass transition temperature (27–30). For large atom displacements (>1 bond length) that change the local bonding configuration, we observed on the order of $\sim 10^{-4}$ displacements/nm² s in the bulk, or roughly one displacement every several images. Because these large atom displacements are relatively rare, we could use the electron beam both as a source for randomly induced structural rearrangements and as a probe to track the material's response.

These techniques produced videos of restructuring, such as the isolated ring rearrangement shown in Fig. 1, B to E, and corresponding movie S1. Such rearrangements are worth examining in detail because they are the building blocks of plastic deformation. Figure 1B shows a high-resolution TEM image of a small region of 2D silica with a cluster of rings initially arranged in a 5-7-5-7 configuration. Each dark spot in the image represents the location of one structural unit: two stacked SiO₄ tetrahedra that are registered out-of-plane. As we continued imaging (Fig. 1, C to E), the 5-7-5-7 cluster transformed into a 6-6-6-6 cluster. Surprisingly, the transformation occurred over several frames. In the 12 s between the stable initial and final configurations, we observed several metastable intermediate states. A similar transformation has been modeled in (11).

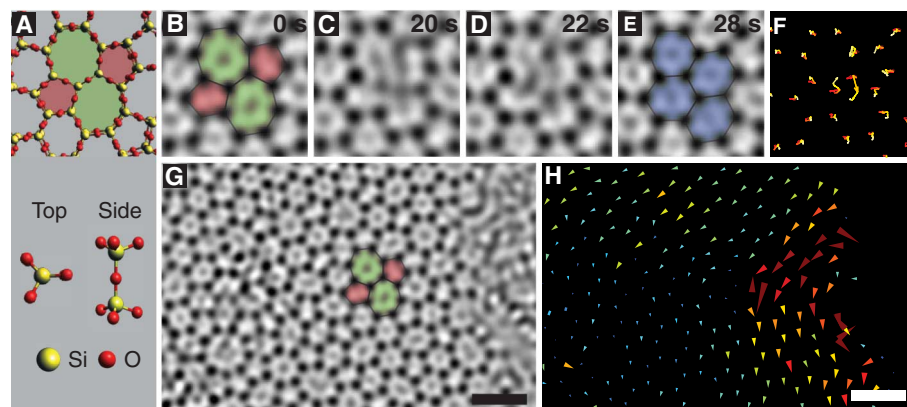


Fig. 1. Elastic and plastic deformation in ring exchange. (A) Cartoon models of the 2D silica structure. (B to E) TEM images showing a ring rearrangement that transforms a 5-7-5-7 cluster into a 6-6-6-6 cluster. The dark spots are Si-O-Si columns that correspond with the top and side views in (A). Images have been smoothed and Fourier-filtered to remove the graphene lattice background [see figs. S2 and S3 and (17)]. (F) A trajectory map of the atomic sites. Color (red to yellow) indicates time of motion. (G) Larger view of the region from (A), and (H) corresponding first-to-last frame displacement map. The arrows have been enlarged $\times 2$ to increase visibility; color indicates size of displacement, from 0 (dark blue) to ≥ 1.3 Å (red). The region between the bond rearrangement and the edge of the sheet exhibits strong local rotation. Scale bars: 1 nm. See also movies S1 and S2.

¹School of Applied and Engineering Physics, Cornell University, Ithaca, NY 14853, USA. ²Electron Microscopy Group of Materials Science, University of Ulm, Ulm 89081, Germany. ³Laboratory of Atomic and Solid State Physics, Cornell University, Ithaca, NY 14853, USA. ⁴Kavli Institute at Cornell for Nanoscale Science, Cornell University, Ithaca, NY 14853, USA.

*These authors contributed equally to this work.

†Corresponding author. E-mail: dm24@cornell.edu

We tracked the position of the atoms over time to measure elastic and plastic deformation in the glass. Each line in Fig. 1F represents the trajectory of a single atomic site in Fig. 1, B to E. We produced these trajectories by cross-correlating our images to remove net motion, fitting Gaussians at each atomic site, and then tracking the atoms from frame to frame using particle-tracking software adapted from colloids research (17, 31). Because 2D silica contains two registered layers of SiO_4 tetrahedra and because Si atoms dominate the TEM image contrast, our methods effectively track the in-plane motion of Si pairs. The two trajectories in the center of Fig. 1F represent plastic deformation: atomic sites that break and form new bonds. We also see the small motions of nearby atoms that move relative to one another while retaining their nearest-neighbor bonding; these are elastic deformations.

The elastic motion of atoms around the ring rearrangement is pronounced on larger length scales. Figure 1G shows an overview image of the bond rearrangement and its proximity to the edge of the silica sheet (right-hand side) (see also movies S1 and S2). Figure 1H shows the corresponding displacement vector field from the first to last frames, a time frame of $\Delta t = 28$ s. The displacement field is dominated by a strong local rotation in the region between the ring exchange and the edge of the sheet.

We examined the correlation between elastic and plastic deformations by analyzing the short-range elastic behavior, as shown in Fig. 2, A to H. Using our tracking data, we took the spatial derivative of the displacement field. This produced the displacement gradient field, which could be separated into its symmetric component, the 2D strain tensor ϵ , and its antisymmetric component, the local rotation matrix ω (32). We plot the magnified, independent experimental components of ϵ and ω in Fig. 2, A to D. These components respectively represent the local volume change, shear, and local rotation. In this analysis, the

motion of the two middle atoms has been removed to isolate the elastic behavior.

To understand these strain components, we conducted molecular dynamics simulations of the ring exchange using the large-scale atomic/molecular massively parallel simulator (LAMMPS) (17, 33). We first produced relaxed structures of 2D silica crystals embedded with either a 5-7-5-7 or 6-6-6-6 cluster. These structures simulate our before-and-after atom configurations and can be processed with the same methods as our experimental images. The resulting ϵ and ω components are shown in Fig. 2, E to H. The agreement between our simulations and experiment provides evidence that our observed elastic displacements are directly correlated to the plastic deformation of the ring exchange and likely represent the relaxation of the structure around the new ring configuration. Our experimental strain fields also match those of an elastic dipole (34), suggesting that even at the atomic scale, continuum elastic mechanics can provide a good model for atomic rearrangements (fig. S4). Finally, the agreement between (amorphous) experiment and (crystalline) simulation suggests that on very short length scales (<1.5 nm), the elastic response of the amorphous material is similar to that of a crystal—in much the same way that the short-range order of amorphous and crystalline materials are also similar.

On larger length scales, however, the elastic deformation of our system deviates from that of an infinite crystal. Figure 2, I to L, shows LAMMPS simulations of displacement fields for a 5-7-5-7 to 6-6-6-6 ring exchange representing four cases: a crystal (Fig. 2I), an amorphous sheet (Fig. 2J), and crystalline and amorphous sheets with an edge (Fig. 2, K and L). These plots separate the key effects contributing to the experimental behavior seen in Fig. 1H. The “crystal” simulation in Fig. 2I displays a highly symmetric displacement field that lacks agreement with our experimental data. In Fig. 2J, adding structural disorder to simulate an “amorphous” structure damps the

symmetry of the strain field and improves agreement with experiment. In Fig. 2, K and L, adding an edge strongly enhances the displacements between the ring exchange and the edge. Figure 2L yields qualitative agreement with the experimental results in Fig. 1H.

In crystals, plastic deformation frequently occurs by introducing and migrating dislocations through the lattice. For amorphous materials, however, this process is far less well understood. One model proposes that deformation in amorphous materials is mediated by shear transformation zones—concentrated, local regions of shear strain that rearrange and interact to produce net deformation (1, 4, 6). Shear transformation zones have been directly observed in colloidal glasses and have been proposed as a model for metallic glasses (6).

Figure 3A shows atomic trajectories in a region undergoing shear, shown in Fig. 3B (see also fig. S5 and movie S3). Shear deformation is apparent when comparing the trajectories of atomic sites in region 1 (top left) and region 3 (bottom right). Although most atoms in region 1 oscillate near their original positions (highlighted in the enlarged atom trajectory and before-and-after bond configuration in Fig. 3C), the atoms in region 3 collectively displace by ~ 2.4 Å relative to region 1 (Fig. 3D). The mechanisms enabling this displacement are visible in regions 2 and 4, which separate the two displacing regions. Both regions 2 and 4 contain a large number of ring rearrangements; one isolated example is shown in Fig. 3E. These rearrangements appear to be mediated by nearby defects: Region 4 is at the edge of the solid, whereas region 2 contains a handful of vacancies (arrows in Fig. 3B). Over the course of the video, this vacancy-induced restructuring gradually changes the bonds connecting regions 1 and 3, enabling their relative displacement. Although the uniform electron beam cannot directly apply shear forces, possible sources of shear stress include forces applied from the edge

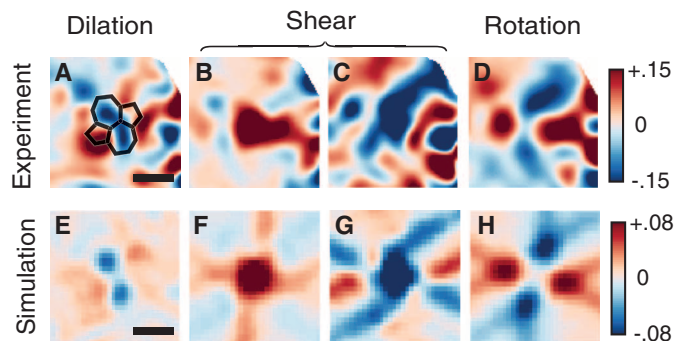
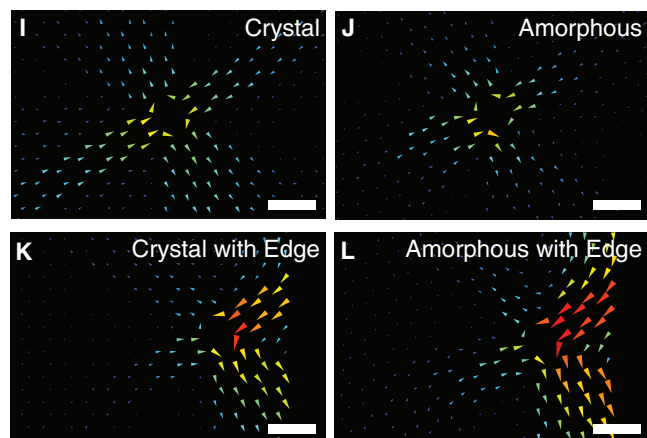


Fig. 2. Strain analysis of 5-7-5-7 to 6-6-6-6 ring exchange. (A to D) Experimental strain components representing (A) the local volume change ($\epsilon_{xx} + \epsilon_{yy}$), (B and C) shear components (ϵ_{xy} and $\epsilon_{xx} - \epsilon_{yy}$), and (D) the local rotation (ω) due to the ring exchange in Fig. 1. Each component is calculated by comparing the position of atoms between the first and last frames, excluding the two atoms at the center of the bond exchange to isolate elastic behavior (17). Overlaid polygons show the original ring configuration. Scale bars: 1 nm. (E to H) LAMMPS simulations for the 5-7-5-7 to 6-6-6-6 ring exchange in crystalline 2D silica. (I to L) Simulated displacement maps isolating the effects of structural and edge disorder. Arrows have been enlarged $\times 4$ for visibility. Color scale is from 0 (dark blue) to ≥ 1 Å (red). Scale bars: 1 nm.



of the sheet by the surrounding material, interactions with the graphene substrate, or internal strain in the disordered silica. Our observation of localized regions of large rearrangements suggests a covalently bonded molecular glass analog for the shear transformation zones seen in other glassy systems.

Figure 4 shows a third class of dynamics that we studied, the rearrangements of atoms at the edge of the 2D sheet. One common way to distinguish solids and liquids is their behavior under stress. Under stress, typical solids exhibit first a linear strain in the elastic regime, followed by a nonlinear stress-strain curve in the plastic regime. This onset of nonlinearity reflects, in part, the activation energy to form and migrate dislocations in crystals, or to nucleate similar rearrangements such as shear transformations in amorphous solids. These rearrangements produce long-range strain

fields. In these respects, the rearrangements observed in the glassy 2D silica discussed above typify the behavior of solids under stress.

In addition to these “solid-like” behaviors that we observed in our 2D glassy phase, we also observed the formation of a second phase, highlighted in blue. This phase is distinguished by its fast mean atomic displacements (>1 bond length between frames) under the electron beam. Importantly, because of its fast rearrangement time scales, this phase is “liquid-like” on the imaging time scales: Under the effective heat bath of the electron beam, it rearranges too quickly to support stresses. These characteristics make our system a nonequilibrium analogy to the equilibrium solid-liquid interface. Electron energy loss spectroscopy confirms that this second phase is composed of oxygen-deficient silica, which has been

shown to rearrange more quickly under electron irradiation than stoichiometric SiO_2 (28). Structurally, this material may be composed of rapidly rearranging 3D amorphous silicon suboxide, monotetrahedral layers of silica, out-of-plane mismatched bitetrahedral layers, or combinations thereof. The structure of this interface is unusual: It resembles neither the sharp, faceted surfaces at crystal/liquid interfaces (35, 36) reported in the literature, nor the gradual evolution in properties that are typical in thermally driven glass/liquid transitions (7, 8). The transition between the two phases occurs over ~ 1 nm (Fig. 4A, on the blue side of the interface).

Figure 4, A to D, shows the time evolution of the interface. In Fig. 4, B to D, “solid-like” regions that have undergone the most displacement relative to their initial configurations are highlighted in red (17). The fastest-moving atoms are concentrated near the edge of the sheet in each frame; here, large sections dissociate and re-form several times over the course of the video, producing new arrangements and sizes of rings with no apparent memory of the previous state. Figure 4E plots the mean squared displacement of atoms in the solid, which increases markedly with proximity to the edge of the sheet for all time intervals measured. This increased motion consists of both increased elastic motion and bond rearrangements of atoms near the edge. An increase in mean displacements at solid/liquid interfaces has also been observed in hard-sphere colloids (37). During the video, the area of the solid and length of the interface fluctuate without increasing or decreasing measurably (fig. S6 and movies S4 and S5), indicating that we are observing the sample near a steady state rather than simply damage-related degradation of the solid.

We have demonstrated a promising avenue for understanding the structure and dynamics of glasses through atomic resolution imaging of 2D glasses. Future work that combines these techniques with well-defined in situ stimuli, such as heating, straining, and electrical biasing, should further extend the potential of these techniques, making it possible to correlate microscopic rearrangements with bulk thermodynamic properties.

References and Notes

1. M. L. Falk, J. S. Langer, *Phys. Rev. E* **57**, 7192–7205 (1998).
2. K. Maeda, S. Takeuchi, *Phys. Status Solidi* **49**, 685–696 (1978) (a).
3. M. L. Falk, C. E. Maloney, *Eur. Phys. J. B* **75**, 405–413 (2010).
4. M. L. Manning, J. S. Langer, J. M. Carlson, *Phys. Rev. E* **76**, 056106 (2007).
5. H. He, M. F. Thorpe, *Phys. Rev. Lett.* **54**, 2107–2110 (1985).
6. P. Schall, D. A. Weitz, F. Spaepen, *Science* **318**, 1895–1899 (2007).
7. W. K. Kegel, A. von Blaaderen, *Science* **287**, 290–293 (2000).
8. E. R. Weeks, J. C. Crocker, A. C. Levitt, A. Schofield, D. A. Weitz, *Science* **287**, 627–631 (2000).
9. A. S. Argon, H. Y. Kuo, *Mater. Sci. Eng.* **39**, 101–109 (1979).
10. P. Y. Huang *et al.*, *Nano Lett.* **12**, 1081–1086 (2012).
11. L. Lichtenstein *et al.*, *Angew. Chem. Int. Ed.* **51**, 404–407 (2012).

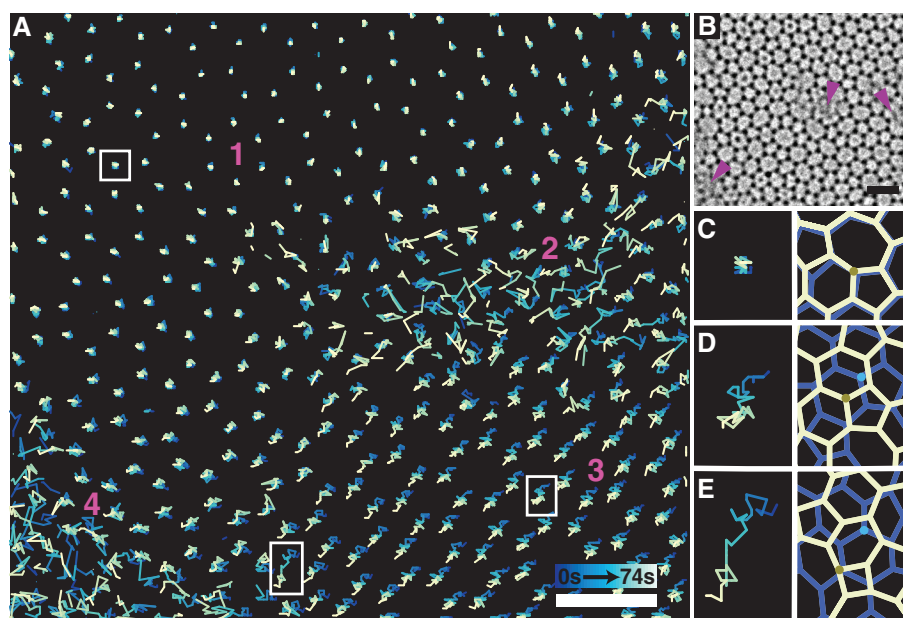


Fig. 3. Imaging and trajectories of shear deformation. (A) Trajectory map over 74 s. The original image is shown in (B), where purple arrows indicate vacancies. The trajectories show four regions with distinct types of motion: oscillation around initial positions (region 1), gradual displacement by ~ 2.4 Å (region 3), and ring rearrangements near vacancies (region 2) or at the edge of the sheet (region 4). The shear strain and bond rearrangements appear directly related: The restructuring in regions 2 and 4 changes the bonds connecting regions 1 and 3, enabling their relative displacement. (C to E) Magnified trajectories (left) and before-and-after bonding configurations (right). (C) oscillates near its original position; (D) moves under shear; and (E) undergoes bond rearrangement. See also movie S3. Scale bars: 2 nm.

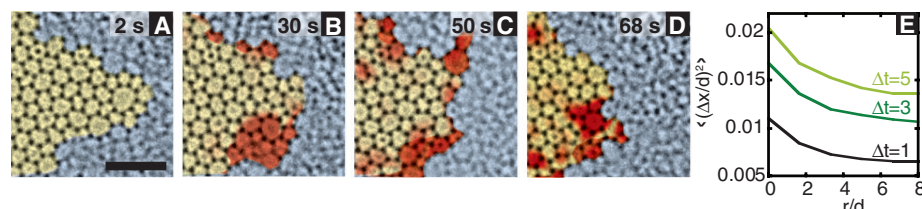


Fig. 4. Restructuring at the edge of the 2D silica sheet. (A to D) Image series of interfacial restructuring. The solid phase is false-colored yellow, with red highlighting the regions of largest rearrangement relative to the first frame (17). The “liquid-like” phase is colored blue. Scale bar: 2 nm. See also movies S4 and S5. (E) Mean squared displacement $\langle (\Delta x/d)^2 \rangle$ of “solid” atoms versus their distance r from the edge for time intervals of $\Delta t = 1, 3$, and 5 s. Both axes are normalized by d , the mean in-plane Si-Si spacing. Atoms near the edge move faster than atoms in the bulk.

12. D. Löffler *et al.*, *Phys. Rev. Lett.* **105**, 146104 (2010).
13. W. H. Zachariasen, *J. Am. Chem. Soc.* **54**, 3841–3851 (1932).
14. L. Lichtenstein, M. Heyde, H.-J. Freund, *Phys. Rev. Lett.* **109**, 106101 (2012).
15. M. Wilson, A. Kumar, D. Sherrington, M. F. Thorpe, *Phys. Rev. B* **87**, 214108 (2013).
16. F. Ben Romdhane *et al.*, *ACS Nano* **7**, 5175–5180 (2013).
17. Materials and methods are available as supplementary materials on Science Online.
18. Z. Lee *et al.*, *Nano Lett.* **9**, 3365–3369 (2009).
19. R. S. Pantelic, J. C. Meyer, U. Kaiser, H. Stahlberg, *Solid State Commun.* **152**, 1375–1382 (2012).
20. E. Nakamura, *Angew. Chem. Int. Ed.* **52**, 236–252 (2013).
21. A. Hashimoto, K. Suenaga, A. Gloter, K. Urita, S. Iijima, *Nature* **430**, 870–873 (2004).
22. J. C. Meyer *et al.*, *Nano Lett.* **8**, 3582–3586 (2008).
23. J. H. Warner *et al.*, *Science* **337**, 209–212 (2012).
24. J. Kotakoski *et al.*, *Phys. Rev. B* **83**, 245420 (2011).
25. J. Kotakoski, A. V. Krashennnikov, U. Kaiser, J. C. Meyer, *Phys. Rev. Lett.* **106**, 105505 (2011).
26. S. Kurasch *et al.*, *Nano Lett.* **12**, 3168–3173 (2012).
27. S. G. Mayr, Y. Ashkenazy, K. Albe, R. S. Averback, *Phys. Rev. Lett.* **90**, 055505 (2003).
28. K. Zheng *et al.*, *Nature Communications* **1**, 24 (2010).
29. L. W. Hobbs, M. R. Pascucci, *J. Phys. Colloq.* **41**, C6-237–C6-242 (1980).
30. P. M. Ajayan, S. Iijima, *Philos. Mag. Lett.* **65**, 43–48 (1992).
31. J. C. Crocker, D. G. Grier, *J. Colloid Interface Sci.* **179**, 298–310 (1996).
32. B. A. Auld, *Acoustic Fields and Waves in Solids* (Wiley, New York, 1973).
33. S. Plimpton, *J. Comput. Phys.* **117**, 1–19 (1995).
34. E. R. Grannan, M. Randeria, J. P. Sethna, *Phys. Rev. B* **41**, 7784–7798 (1990).
35. J. M. Howe, H. Saka, *MRS Bull.* **29**, 951–957 (2004).
36. S. E. Donnelly *et al.*, *Science* **296**, 507–510 (2002).
37. J. Hernández-Guzmán, E. R. Weeks, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 15198–15202 (2009).

Acknowledgments: The raw data presented in this work are available in the supplementary movies. S.K. acquired, aligned, and preprocessed the TEM data under the supervision of U.K. P.Y.H. and J.S.A. designed and conducted the atom tracking and strain and deformation analysis under the supervision of D.A.M. and P.L.M. A.S., A.A.A., and J.P.S. contributed theoretical understanding via continuum mechanics analysis and LAMMPS simulations. P.Y.H., J.S.A., and D.A.M. wrote the paper. All authors contributed to the discussion of results and commented on and edited the paper. This work was supported by the NSF through the Cornell Center for Materials Research (NSF DMR-1120296).

Additional support for P.Y.H. was provided by the NSF Graduate Research Fellowship Program under grant DGE-0707428. A.A.A., A.S., and J.P.S. were supported by NSF grant PHY-0941095. J.S.A. and P.L.M. were supported by the Air Force Office of Scientific Research through the Graphene MURI (FA9550-09-1-0691 and FA9550-10-1-0410). U.K. and S.K. acknowledge support from the Deutsche Forschungsgemeinschaft (German Research Foundation) and the Ministry of Science, Research and the Arts (MWK) of Baden-Württemberg through the Sub Angstrom Low-Voltage Electron Microscopy project. The authors acknowledge discussions with M. K. Bles, I. Cohen, S. J. Gerbode, R. Hovden, J. Kotakoski, A. V. Krashennnikov, B. Leahy, Y.-C. Lin, M. L. Manning, E. R. Weeks, and A. M. van der Zande. We thank A. Srivastava, V. Skakalova, and J. Smet from the Max Planck Institute for Solid State Research in Stuttgart, Germany, for the sample. Microscopy support and maintenance were provided by J. Biskupek.

Supplementary Materials

www.sciencemag.org/content/342/6155/224/suppl/DC1

Materials and Methods

Figs. S1 to S6

Movies S1 to S5

References (38–40)

21 June 2013; accepted 26 August 2013

10.1126/science.1242248

Waveform Tomography Reveals Channeled Flow at the Base of the Oceanic Asthenosphere

Scott French,¹ Vedran Lekic,² Barbara Romanowicz^{1,3,4*}

Understanding the relationship between different scales of convection that drive plate motions and hotspot volcanism still eludes geophysicists. Using full-waveform seismic tomography, we imaged a pattern of horizontally elongated bands of low shear velocity, most prominent between 200 and 350 kilometers depth, which extends below the well-developed low-velocity zone. These quasi-periodic fingerlike structures of wavelength ~2000 kilometers align parallel to the direction of absolute plate motion for thousands of kilometers. Below 400 kilometers depth, velocity structure is organized into fewer, undulating but vertically coherent, low-velocity plumelike features, which appear rooted in the lower mantle. This suggests the presence of a dynamic interplay between plate-driven flow in the low-velocity zone and active influx of low-rigidity material from deep mantle sources deflected horizontally beneath the moving top boundary layer.

Mantle convection is responsible for driving plate motions on Earth, but the detailed morphology of convection patterns remains unresolved. Because seismic velocities are affected by temperature, and seismic anisotropy is affected by alignment of crystals, seismic tomography can be used to map the patterns of flow in the earth's mantle. Global seismic mantle tomography has provided important constraints on the long-wavelength shear-velocity structure, highlighting in particular the correlation of velocity patterns in the top 200 km with surface tectonics and documenting the widespread presence of the low-velocity zone (LVZ) under ocean basins.

Likewise, the presence of two antipodal large low-shear-velocity provinces (LLSVPs) at the base of the mantle under the central Pacific and Africa is a robust feature of all tomographic models (1). Hotspots appear to be located preferentially above the LLSVPs (2) or on their borders (3). There is also a striking correlation at long wavelengths between the location of the LLSVPs and high attenuation in the mantle transition zone (4). However, plume conduits (5, 6) and roll-like secondary convection patterns (7) remain difficult to image tomographically.

We used full-waveform inversion, coupled with synthetic seismogram computation using the Spectral Element Method, to image global radially anisotropic shear-velocity (V_S) structure at upper-mantle and transition-zone depths. This approach is well suited to remedy the known limitations of classical tomographic techniques (8), as already demonstrated at the local (9) and regional (10) scales. Our second-generation global

model, SEMum2, refines an earlier one developed by our group (11) and in particular includes a more realistic crust (supplementary text and figs. S1 to S4). Compared with other global shear-velocity models (figs. S5 to S8), SEMum2 more accurately recovers both the depth and strength of the low-velocity minimum under ridges. It also shows stronger velocity minima in the LVZ, a more continuous signature of fast velocities in subduction zones, and stronger, clearly defined, low-velocity “conduits” under the Pacific Superswell (12) while confirming the robust long-wavelength structure imaged in previous studies (supplementary text S2.3 and figs. S7 and S8), such as the progressive weakening and deepening of the oceanic LVZ with overlying plate age.

Cluster analysis (13) of V_S profiles in the depth range 30 to 350 km in SEMum2 (supplementary text S3) provides an objective way to analyze the model and isolates an anomalously low-velocity region—most prominent in the depth range 200 to 350 km although also reflected in the overlying LVZ (Fig. 1, A and B, and fig. S9), organized in elongated bands, and clearest on the Pacific plate (Fig. 1A), where it spans from ~100 million-year-old ocean floor to the East Pacific Rise (EPR). In a map view of SEMum2 at a depth of 250 km (Fig. 2A), these prominent structures appear as fingerlike zones of significantly slower-than-average V_S (~3 to 4%). They are also present under other plates: off west Antarctica, in some parts of the North and South Atlantic and western Indian Oceans, and possibly in the southwestern part of the Australian plate (Fig. 2A and fig. S10). These fingerlike structures are not only well-resolved in the SEMum2 model but also are robust with respect to estimated model uncertainties, are compatible with independent waveform data, and cannot be explained by unmapped azimuthal anisotropy in our inversion (supplementary text S4 and figs. S13 to S17).

¹Berkeley Seismological Laboratory, 209 McCone Hall, Berkeley, CA 94720, USA. ²Department of Geology, University of Maryland, College Park, MD 20742, USA. ³Collège de France, 11 Place Marcelin Berthelot, 75005 Paris, France. ⁴Institut de Physique du Globe de Paris, 1 rue Jussieu, 75238 Paris Cedex 05, France.

*Corresponding author. E-mail: barbara@seismo.berkeley.edu

We find that these low-velocity fingers (LVFs) are oriented subparallel to the direction of absolute plate motion (APM) (Fig. 2B and supplementary text S5.1) (14). Perpendicular to the APM, the alternating zones of very low and somewhat higher-than-average V_S have a wavelength of ~ 2000 km, as illustrated in depth cross-sections on the Pacific Plate (Fig. 3, B and C). This wavelength corresponds to a peak in power in the geoid, as determined with directional wavelet analysis, which is also aligned with the direction of the APM (fig. S11 and supplementary text 5.3) (15). In cross-section parallel to the APM, the contrast in structure at depth within and adjacent to the LVFs is very clear (Fig. 3, D and E). The LVFs extend for many thousands of kilometers and reach beneath the conventional LVZ, which bottoms at an approximately constant depth of ~ 150 to 200 km (Fig. 3D). Below 200 km, velocities are as low within the LVFs as they are between fingers in the LVZ, despite the greater depth (Fig. 3). In contrast, the EPR itself is a shallow feature in isotropic V_S (fig. S6) but stands out in radial anisotropy as a zone where $V_{SV} > V_{SH}$ in the depth range 150 to 300 km (fig. S12C). This indicates that dominantly horizontal flow in the LVFs away from the ridge transitions to dominantly vertical flow under the ridge. Although local minima in the LVZ are associated with the LVFs, the strongest minima in the LVZ appear under ridges (Fig. 3 and fig. S12).

Such alternating zones of high and low velocities have previously been found along the Fiji-Hawaii corridor (16), and an elongate band of low velocities, within a similar depth range to the LVFs, has recently been imaged in the south Atlantic (17). At a smaller scale, tomographic maps based on the PLUME experiment (18, 19) show a zone of fast velocities surrounding Hawaii, particularly strong in V_S to the southwest near 300 km depth. In our model, this corresponds to a domain of higher velocities between LVFs. Our study thus ties together these isolated observations, suggesting that they are manifestations of a single, consistent, large-scale pattern of LVFs (Figs. 2 and 3) aligned with the APM, present in the oceans worldwide, and extending in a narrow depth range below the LVZ.

At the global scale, many of the fingers underlie regions associated with hotspot tracks or seamount chains: for example, in the northwest Atlantic, the New England seamount chain, in the South Atlantic, the Walvis ridge, or the Cape Verde track, and in the Indian Ocean, portions of the Reunion hotspot track (Fig. 2 and fig. S10). In contrast to the top 300 km, deeper V_S structure in the region spanning from the Pacific Superswell to Hawaii is characterized by vertically elongated plumelike conduits (Fig. 4). Not all LVFs are connected to the conduits below, and the latter are not straight, but meander with depth and appear to be rooted in the lower mantle. The main hotspots in the central Pacific are located generally in the vicinity of the deep conduits but not immediately above them (Fig. 4D).

Although the resolution of our modeling enables the detection of the stronger mantle upwellings, such as those beneath Hawaii and the Superswell, the actual plume conduits could be narrower,

and other, weaker ones, may not yet be resolved and will necessitate modeling at shorter periods (6). The absence of a direct vertical correspondence between hotspot locations and the imaged

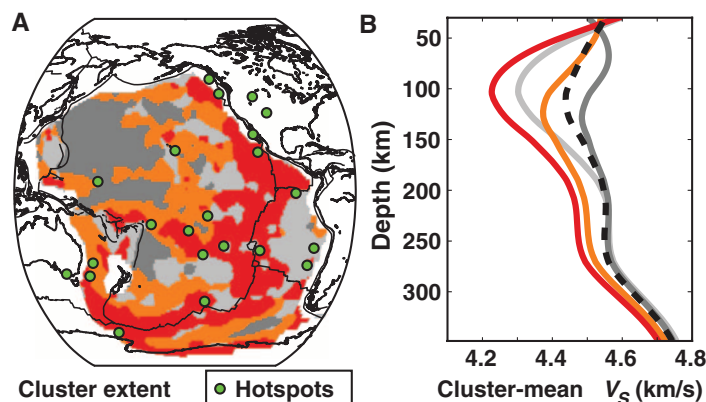


Fig. 1. Cluster analysis of oceanic upper-mantle shear velocity. (A) Pacific portion of a global cluster analysis (13) of model SEMum2 assuming four regions in the oceans (supplementary text S3). (B) Corresponding average depth profiles in each of the four regions (global average also shown, black dashed line).

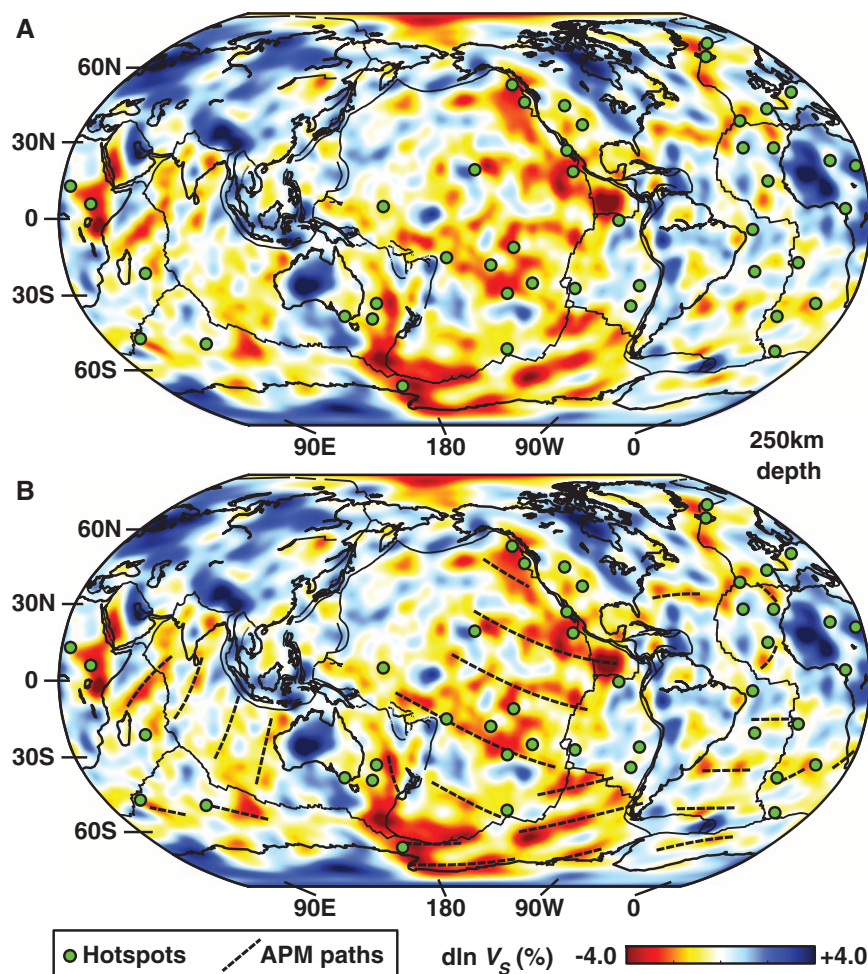


Fig. 2. Shear-velocity structure and absolute plate motion. (A) Map view of relative isotropic V_S variations (percent) from the mean at 250 km. (B) Identical to (A) but with plate-motion streamlines (broken lines) from the APM model of Kreemer (14). Green circles denote hotspot locations from Steinberger (30).

plumes suggests a complex interaction of the upwelling flow with the lithosphere (20). Above ~350 km depth, two interacting structural patterns appear to dominate: (i) the increasing depth and decreasing strength of the LVZ as a function of age in the depth range 50 to 200 km (Fig. 1B) and (ii) the difference in velocity—and therefore likely temperature and/or composition, as well as viscosity—within and outside of LVFs (Figs. 1B and 3 and supplementary text S5.2). In some locations, the LVFs appear to feed from the quasi-vertical conduits, suggesting deflection and channeling in the asthenosphere of active upwelling from low-viscosity plumes—similar to viscous fingering experiments in which a low-viscosity fluid is injected between two rigid horizontal plates or stratified, higher-viscosity fluids (21). This horizontally deflected flow then aligns in the direction of plate motion, driven by a combination of asthenospheric return flow (22, 23) and upwelling-induced flow directed toward pressure minima at ridges (24–26). The pattern of radial anisotropy in the vicinity of the ridge (fig. S12C) further supports active ridge-ward flow in these channels.

Active influx from deep upwellings deflected toward the ridge may be enhanced by flow in a narrow low-viscosity layer (27). The absence of any distinct deeper low-velocity structure beneath ridges (fig. S6), and the fact that some of the LVFs terminate at ridges (such as the Antarctic plate), confirms the passive nature of mantle upwelling beneath ridges.

Whether or not these observations can be explained by viscous fingering and channelization alone or in combination with other phenomena, such as secondary convection, is unknown. Other studies have described evidence for viscous fingering on the Pacific plate, aligned with the plate motion, albeit at an order-of-magnitude smaller spatial scale than seen here (23). The width of the fingers we observed (~1000 km) is large compared with the thickness of the channel (up to 350 km), whereas typical scaling in laboratory or numerical fingering experiments obtain a width-to-thickness ratio of ~2 (21). Secondary convection in the form of Richter rolls occurs with a horizontal-to-vertical scaling of 1 [albeit in a constant-viscosity fluid (7), a condition quite different from that in the Earth] but has

previously been sought in the upper mantle at smaller scales than seen here (28). The LVFs are observed both below the fast spreading Pacific plate, where roll-like secondary convection may be expected, and below slow-moving plates [for example, the Antarctic plate (Fig. 2 and fig. S18) and the Atlantic Ocean (fig. S19)], where Richter rolls are unlikely to form. Comparison of the LVFs and Pacific geoid undulations with the same orientation and wavelength (fig. S11) (15) may also provide insight into causative dynamics. Indeed, in a simple Richter-like secondary convection scenario one would expect the bands of quasi-APM orientation in the geoid to be aligned vertically with the up- (LVF) and down-welling (inter-LVF) limbs. Instead, the LVFs fall at the edges or between these features—an observation more consistent with the presence of channelized flow (supplementary text S5.3). A further clue as to the nature and origin of the global pattern of LVFs that we document here might come from the geochemistry of mid-ocean ridge basalts, whose long-wavelength isotopic anomalies fluctuate with a similar pattern along the mid-Atlantic Ridge (29).

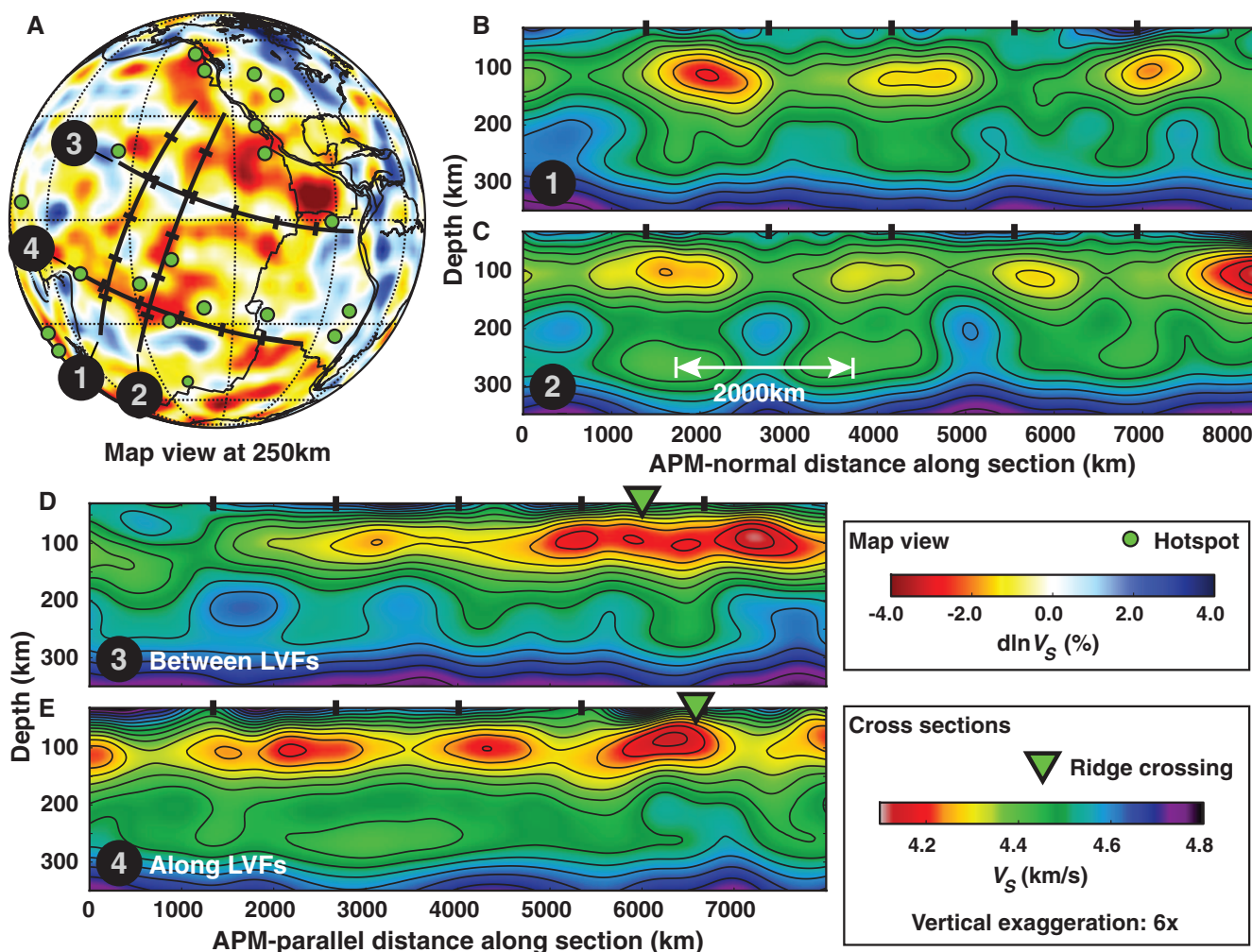


Fig. 3. Low-velocity fingers in cross-section. (A) Map view of relative isotropic V_s variations at 250 km depth, with location of depth profiles shown in (B) to (E). (B and C) Absolute variations in isotropic V_s along two profiles

perpendicular to the APM. (D and E) Same as (B) and (C) for profiles parallel to the APM: (D) between two LVFs and (E) along an LVF. Green circles in (A) denote hotspots of Steinberger (30).

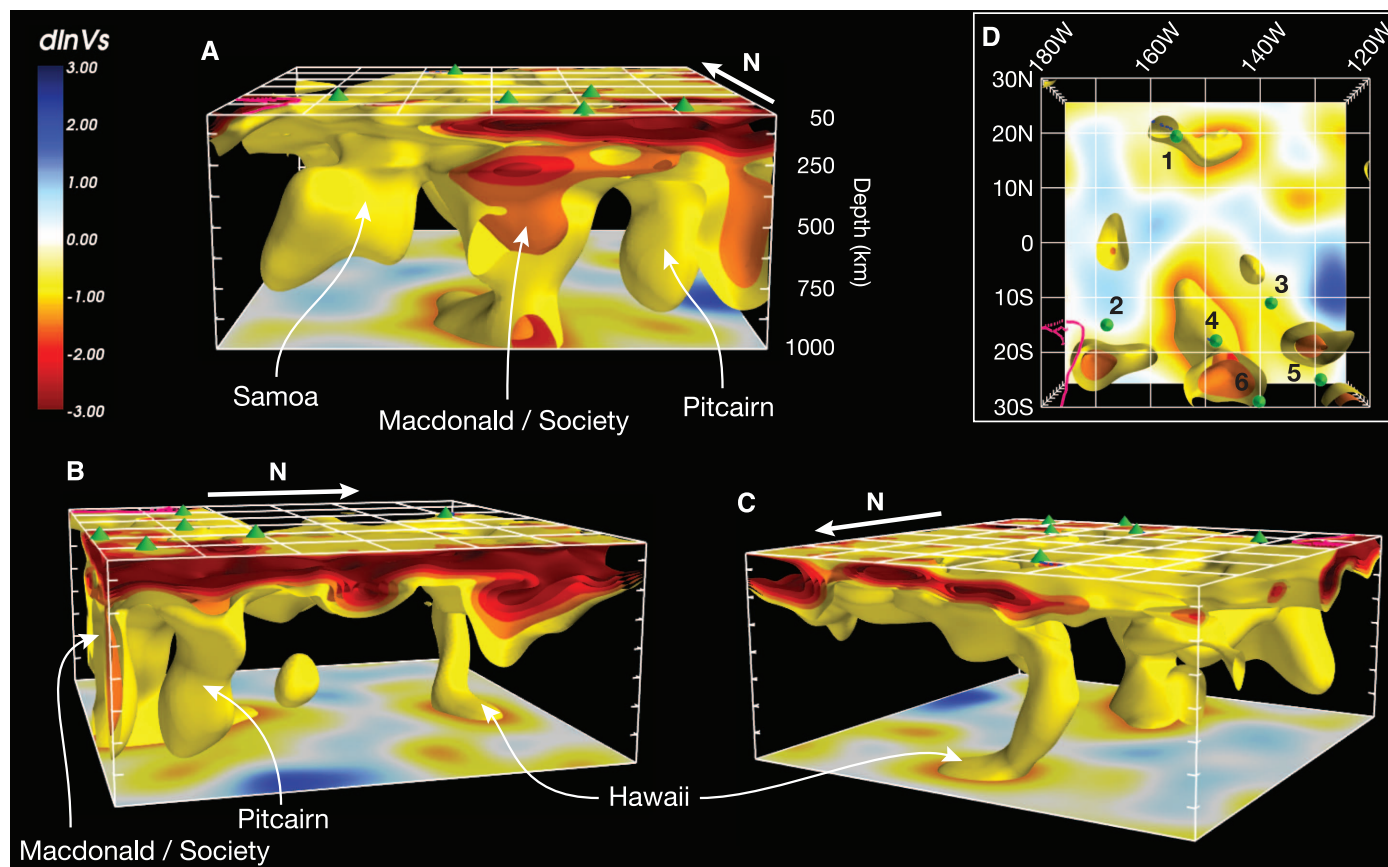


Fig. 4. Three-dimensional rendering of SEMum2 shear-velocity structure. Isotropic relative V_S variations in the central Pacific region (from the Superswell north to Hawaii) viewed from (A) the south, (B) the east and (C) the north. Minimum and maximum isosurface levels are -3% and -1% , respectively. In (A), the LVZ becomes thinner to the west, and the LVF disappears at the Tonga-Fiji subduction zone. In (B), the LVFs appear clearly separated from one another in the direction perpendicular to the APM. The absence of pronounced horizontally elongated low velocities below 200 km depth between fingers is visible in (C). Below 300 to 400 km, the low ve-

locities are organized into predominantly vertical plume-like features. In particular, the Hawaiian "plume" appears east of Hawaii at the bottom of our model (1000 km depth) then turns to the northwest, before being deflected eastward again just below the LVF [(B) and (C)]. (D) View from the top shows the geographic location of the box and major hotspots in relation to the low-velocity conduits, rendered here at 500 km depth: 1, Hawaii; 2, Samoa; 3, Marquesas; 4, Tahiti; 5, Pitcairn; and 6, Macdonald. The magenta outline indicates the location of the Tonga-Fiji subduction zone. Hotspot locations are those of Steinberger (30).

References and Notes

- V. Lekić, S. Cottaar, A. M. Dziewonski, B. Romanowicz, *Earth Planet. Sci. Lett.* **357–358**, 68–77 (2012).
- M. Richards, D. Engebretson, *Nature* **355**, 437–440 (1992).
- A. Davaille, E. Stutzmann, G. Silveira, J. Besse, V. Courtillot, *Earth Planet. Sci. Lett.* **239**, 233–252 (2005).
- B. Romanowicz, Y. C. Gung, *Science* **296**, 513–516 (2002).
- W. J. Morgan, *Nature* **230**, 42–43 (1971).
- F. Rickers, A. Fichtner, J. Trampert, *Geophys. J. Int.* **190**, 650–664 (2012).
- F. M. Richter, B. Parsons, *J. Geophys. Res.* **80**, 2529–2541 (1975).
- G. Nolet, F. A. Dahlen, *J. Geophys. Res.* **105**, (B8), 19043–19054 (2000).
- C. Tape, Q. Liu, A. Maggi, J. Tromp, *Science* **325**, 988–992 (2009).
- A. Fichtner, B. L. N. Kennett, H. Igel, H.-P. Bunge, *Geophys. J. Int.* **179**, 1703–1725 (2009).
- V. Lekić, B. Romanowicz, *Geophys. J. Int.* **185**, 799–831 (2011a).
- M. K. McNutt, K. M. Fischer, in *Seamounts, Islands and Atolls*, Geophysical Monographs Series 43, B. H. Keating et al., Eds. (AGU, Washington D. C., 1987), pp. 25–34.
- V. Lekić, B. Romanowicz, *Earth Planet. Sci. Lett.* **308**, 151–160 (2011b).
- C. Kreemer, *J. Geophys. Res.* **114**, B10405 (2009).
- M. Hayn et al., *Geophys. J. Int.* **189**, 1430–1456 (2012).
- R. Katzman, L. Zhao, T. H. Jordan, *J. Geophys. Res.* **103**, 17933–17971 (1998).
- L. Colli, A. Fichtner, H.-P. Bunge, *Tectonophysics*, published online 27 June 2013 (10.1016/j.tecto.2013.06.015).
- C. J. Wolfe et al., *Earth Planet. Sci. Lett.* **303**, 267–280 (2011).
- G. Laske et al., *Geophys. J. Int.* **187**, 1725–1742 (2011).
- J. M. O'Connor et al., *Nat. Geosci.* **5**, 735–738 (2012).
- D. Snyder, S. Tait, *J. Fluid Mech.* **369**, 1–21 (1998).
- J. Phipps Morgan, W. J. Morgan, Y.-S. Zhang, W. H. F. Smith, *J. Geophys. Res.* **100**, (B7), 12,753–12,767 (1995).
- N. Harmon, D. W. Forsyth, D. S. Weeraratne, Y. Yang, S. C. Webb, *Earth Planet. Sci. Lett.* **311**, 306–315 (2011).
- D. R. Toomey et al., *Earth Planet. Sci. Lett.* **200**, 287–295 (2002).
- J. K. Hillier, A. B. Watts, *J. Geophys. Res.* **109**, B10102 (2004).
- M. D. Ballmer, C. P. Conrad, E. I. Smith, N. Harmon, *Geology* **41**, 479–482 (2013).
- T. Höink, A. Lenardic, M. Richards, *Geophys. J. Int.* **191**, 30–41 (2012).
- M. D. Ballmer, J. van Hunen, G. Ito, T. A. Bianco, P. J. Tackley, *Geochim. Geophys. Res.* **10**, GC002386 (2009).
- A. W. Hofmann, in *Treatise on Geochemistry Update 1*, H. D. Holland, K. K. Turekian, Eds. (Elsevier, New York, 2007), vol. 2.03, pp. 1–44.
- B. Steinberger, *J. Geophys. Res.* **105**, 11,127–11,152 (2000).

Acknowledgments: We thank A. Davaille, C. Jaupart, and D. Shim for helpful discussions. This work was supported by the National Science Foundation (NSF) (grant EAR-0738284). S.F. acknowledges support from a NSF Graduate Research Fellowship. Our waveform data set was collected from the Incorporated Research Institutions for Seismology (www.iris.edu). Computations were performed at the National Energy Research Scientific Computing Center (supported by the U.S. Department of Energy Office of Science, contract DE-AC02-05CH11231). This is Berkeley Seismological Laboratory contribution 13-13. The SEMum2 model is available at <http://seismo.berkeley.edu/~sfrench/SEMum2>.

Supplementary Materials

www.sciencemag.org/content/342/6155/227/suppl/DC1
Supplementary Text
Figs. S1 to S19
Table S1
References (31–60)

17 April 2013; accepted 26 August 2013
Published online 5 September 2013;
10.1126/science.1241514

RNA Interference Functions as an Antiviral Immunity Mechanism in Mammals

Yang Li,^{1*} Jinfeng Lu,^{1,2*} Yanhong Han,¹ Xiaoxu Fan,¹ Shou-Wei Ding^{1,2†}

Diverse eukaryotic hosts produce virus-derived small interfering RNAs (siRNAs) to direct antiviral immunity by RNA interference (RNAi). However, it remains unknown whether the mammalian RNAi pathway has a natural antiviral function. Here, we show that infection of hamster cells and suckling mice by Nodamura virus (NoV), a mosquito-transmissible RNA virus, requires RNAi suppression by its B2 protein. Loss of B2 expression or its suppressor activity leads to abundant production of viral siRNAs and rapid clearance of the mutant viruses in mice. However, viral small RNAs detected during virulent infection by NoV do not have the properties of canonical siRNAs. These findings have parallels with the induction and suppression of antiviral RNAi by the related Flock house virus in fruit flies and nematodes and reveal a mammalian antiviral immunity mechanism mediated by RNAi.

RNA interference (RNAi) acts as a natural antiviral defense in plants, insects, nematodes, and fungi; accordingly, virulent infection in these organisms requires suppression of antiviral RNAi by a virus-encoded suppressor of RNAi (VSR) (1–12). Induction of

antiviral RNAi depends on the processing of virus-specific double-stranded RNA (dsRNA) by Dicer nuclease into 21- to 24-nucleotide (nt) small interfering RNAs (siRNAs), which are short dsRNAs with two unpaired nucleotides at the 3' end of either strand (1–9). Mammalian viral mRNAs are as susceptible as cellular mRNAs to RNAi programmed by synthetic siRNAs, and virus-derived small RNAs (vsRNAs) are found in mammalian cells infected by RNA viruses (9, 13, 14). Mammalian viral proteins that can suppress insect and plant RNAi or artificially induced RNAi in mammalian cells have been iden-

tified, and the virulence function of one such protein can be complemented by distinct siRNA-sequestering plant VSRs (9, 15–19). However, it remains unknown whether virus infection triggers production of canonical viral siRNAs in mammals or if mammalian virus infections require specific suppression of an antiviral RNAi response (9).

Nodamura virus (NoV) is mosquito-transmissible, highly virulent to suckling mice and suckling hamsters, and belongs to the same bipartite positive-strand RNA virus genus as Flock house virus (FHV), an insect pathogen (20). FHV infection in *Drosophila* requires expression of its VSR protein B2, a dsRNA-binding protein, to inhibit Dicer processing of dsRNA viral replication intermediates into siRNAs (3, 12, 21–24). Clearance of a B2-deficient FHV mutant in cultured *Drosophila* cells is therefore associated with abundant accumulation of viral siRNAs (24). Because the B2 ortholog of NoV exhibits similar in vitro VSR activities and suppresses experimental RNAi in mammalian cells (15, 16, 24), we reasoned that use of NoVΔB2, a B2-deficient mutant of NoV (25), to challenge baby hamster kidney 21 (BHK-21) cells might facilitate detection of mammalian viral siRNAs. In two independent experiments, we compared deep sequencing profiles of 18- to 28-nt small RNAs from BHK-21 cells 2 or 3 days postinoculation (dpi) with either NoV or NoVΔB2. In cells infected by NoV, vsRNAs were highly abundant, but they displayed an overwhelming bias for positive strands (~97%), showed no size preference expected for Dicer products

¹Department of Plant Pathology and Microbiology, and Institute for Integrative Genome Biology, University of California, Riverside, CA 92521, USA. ²Graduate Program in Genetics, Genomics, and Bioinformatics, University of California, Riverside, CA 92521, USA.

*These authors contributed equally to this work.

†Corresponding author. E-mail: shou-wei.ding@ucr.edu

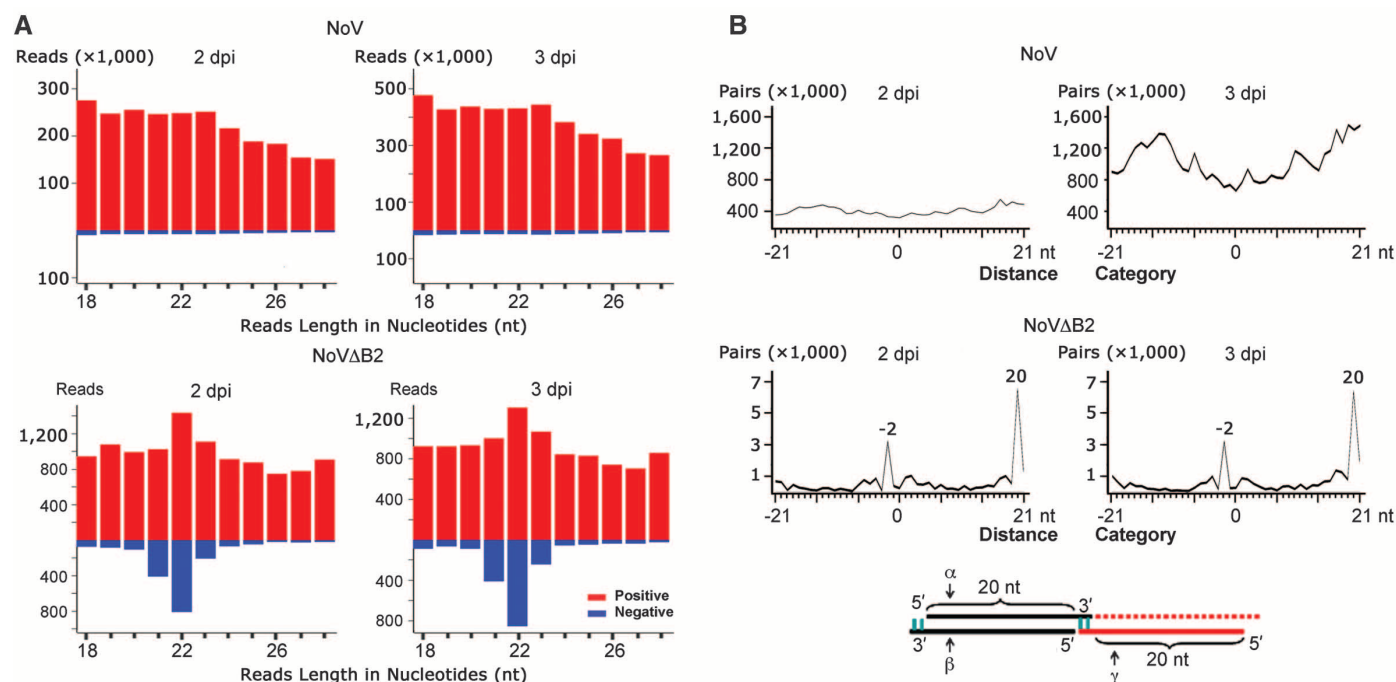


Fig. 1. siRNA properties of vsRNAs in BHK-21 cells. (A) Length distribution and abundance of positive- or negative-strand vsRNAs from cells 2 or 3 dpi with NoV or NoVΔB2. (B) Total counts of pairs of complementary 22-nt vsRNAs of NoV or NoVΔB2 in each distance category (in

nucleotides) between 5' and 3' ends of a complementary vsRNA pair, defined as 0 for perfect base-paired 22-nt vsRNAs with blunt ends, –2 for pairs with 2-nt overhang at the 3'-end of each strand (α and β), or 20 for pairs with 20-nt overhang at the 5'-ends (α and γ).

(Fig. 1A and table S1), and are likely breakdown products from the abundant positive-strand viral RNAs (9).

By contrast, vsRNAs from NoVΔB2-infected cells were much less abundant and exhibited reduced positive-strand bias (~85%) (table S1). Notably, ~77% of the total negative-strand vsRNA reads in both libraries were in the 21- to 23-nt size range with a major 22-nt peak, similar to Dicer-dependent cellular microRNAs (Fig. 1A and fig. S1A). The unique negative-strand vsRNAs also had a dominant 22-nt peak (fig. S1B). Therefore, NoVΔB2 vsRNAs display patterns of length distribution and strand bias expected for Dicer products as found for plant and invertebrate viral siRNAs (9).

NoVΔB2 vsRNAs exhibited properties of canonical siRNAs (Fig. 1B and table S1). First, both NoVΔB2 libraries were enriched for a population of 22-nt vsRNAs that contained a 20-nt perfectly base-paired duplex region with 2-nt 3' overhangs (Fig. 1B, peak “-2” and siRNAs α/β). Enrichment for 22-nt canonical siRNA pairs was not found for the comparably much more abundant vsRNAs of NoV (Fig. 1B). Second, we detected a more dominant population of complementary 22-nt vsRNA pairs with 20-nt 5'-end overhangs only for NoVΔB2 vsRNAs (Fig. 1B, peak “20” and siRNAs α/γ). These findings together suggest Dicer-dependent processing of the same viral dsRNA precursor into successive 22-nt viral siRNA duplexes in cells infected by NoVΔB2, but not by NoV.

In contrast to the efficient infection of BHK-21 cells by B2-expressing NoV (25), NoVΔB2 maintained infection only at low levels (Fig. 2). Higher accumulation levels of NoVΔB2 were restored (Fig. 2), however, in BHK-21 cells engi-

neered with a stably expressed transgene encoding either NoV B2 or Ebola virus virion protein 35 (VP35), the latter of which suppresses experimental RNAi in mammalian cells by a distinct mechanism (26, 27). These results show that RNAi suppression by a cognate or heterologous VSR expressed from either the viral genome or an ectopic transgene is essential for robust virus infection in mammalian cells. We conclude therefore that NoVΔB2 is defective only in RNAi suppression, and the RNAi response induced by NoVΔB2, characterized by the production of viral siRNAs, has potent antiviral activity in BHK-21 cells. Consistently, rescue of NoVΔB2 infection was also observed in RNAi-defective mouse embryonic stem cells deleted of all Argonaute genes (28).

NoV is lethal to 7-day-old mice when injected intraperitoneally (29). Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis validated the spread of both NoV and NoVΔB2 from the injected abdominal cavity to the fore- and hindlimb tissues 24 hours after inoculation (Fig. 3A). The difference between NoV and NoVΔB2 accumulation levels was small at 1 dpi, although higher doses of NoVΔB2 were inoculated into each mouse (supplementary materials), but became progressively more pronounced at later infection times. By 4 dpi, NoV RNA levels were comparable to those of ribosomal RNAs (rRNAs), whereas the accumulation of NoV was more than 1000 times that of NoVΔB2 (Fig. 3, A and B). Accordingly, unlike the 100% mortality observed 5 days post-NoV infection, suckling mice challenged by NoVΔB2 remained healthy for the duration of the experiment, up to 4 weeks postinoculation (Fig. 3C). Our quantitative RT-PCR analysis on the expression of 84 key genes

from the known innate antiviral pathways (30) in suckling mice at 4 dpi detected no major differences between infection by NoVΔB2 and NoV (table S2). This suggested that rapid in vivo clearance of NoVΔB2 was not mediated by one of the known innate antiviral pathways. Moreover, we found that a NoV mutant (NoVmB2) carrying a single Arg to Gln mutation at position 59 of B2, known to abolish B2's VSR activity in vitro (3, 24), was as nonvirulent as NoVΔB2 in suckling mice and was also progressively cleared from 4 dpi (Fig. 3A). Thus, in vivo infection and virulence of NoV require the RNAi suppressor activity of B2.

Northern blot hybridization detected accumulation of discrete species of viral siRNAs in NoVΔB2-inoculated suckling mice (Fig. 3D, right panel), as found in plant and invertebrate hosts after virus infection (1, 2, 9). The mouse viral siRNAs migrated as a dominant 22-nt band alongside a weaker, 21-nt signal and became detectable at 2 dpi and remained so up to 7 dpi even through the accumulation of NoVΔB2 was low at both 2 and 7 dpi (Fig. 3, A and B). In contrast, vsRNAs from NoV-infected mice appeared as bands of heterogeneous sizes (Fig. 3D, right panel). Notably, the 22-nt viral siRNAs became readily detectable in suckling mice inoculated with NoVmB2 (Fig. 3D, left panel). Therefore, rapid virus clearance resulting from loss of viral suppression of RNAi in NoVΔB2- and NoVmB2-infected mice was consistently accompanied with abundant production of the 22-nt viral siRNAs.

We further deep sequenced small RNAs from suckling mice 4 days after NoV inoculation and from those 1 or 2 days after NoVΔB2 inoculation. NoV vsRNAs showed no size preference, and

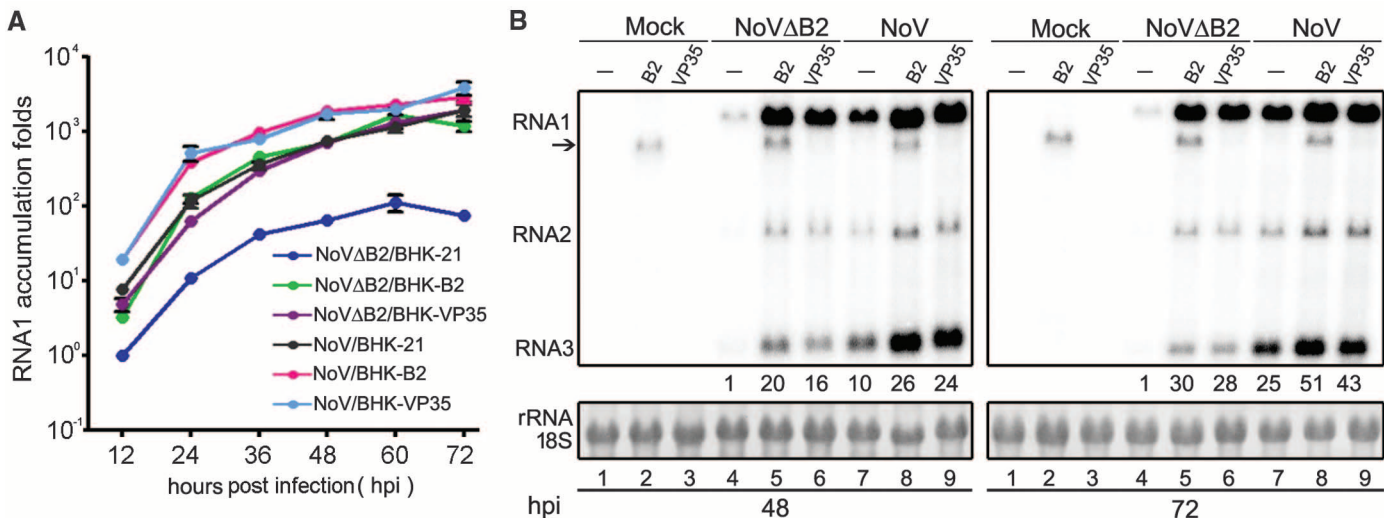


Fig. 2. NoV infection requires RNAi suppression. (A) BHK-21 cells or BHK cells stably expressing B2 or VP35 were mock-infected or infected by NoVΔB2 or NoV of the same titer. Every 12 hours postinfection (hpi), the viral genomic RNA1 levels were determined by quantitative RT-PCR with the accumulation level of NoVΔB2 in BHK-21 cells at 12 hpi set as 1. Error bars

indicate standard deviation of three replicates. (B) Accumulation of NoV and NoVΔB2 RNAs 1 to 3 in the infected cells detected by Northern blotting. RNA1 signal quantified by phosphorimaging was shown with that of NoVΔB2 in BHK-21 cells (lanes 4) set as 1. Detection of B2 transgene mRNA (arrow) was visible. 18S rRNA staining served as loading control.

the 22-nt vsRNAs of NoV were not enriched for canonical siRNAs (Fig. 4, A and B), which suggested B2 suppression of viral siRNA biogenesis in NoV-infected mice. We noted that NoV vsRNAs cloned from mice contained more abundant negative strands (16%) than those from cell culture (3%) (table S1), which might indicate weak *in vivo* dicing of NoV dsRNA in the presence of B2. In contrast, ~85% of NoV Δ B2 small RNAs from mice were 21- to 23-nt long, with 22 nt as the predominant size for both strands (Fig. 4A and fig. S1B). A higher density of viral siRNAs was found to target the RNA3-transcribing region of RNA1 and the 5'-terminal region of RNAs 1 and 2 in NoV Δ B2-infected mice and BHK-21 cells (Fig. 4C). The relative abundance of viral siRNAs in NoV Δ B2-infected mice (0.3%) (table S1) was similar to that found in fruit flies (0.5 to 0.9%) undergoing FHV Δ B2 clearance (12). NoV Δ B2 siRNAs from mice at 2 dpi were divided approximately equally into positive and

negative strands (Fig. 4A), and 65% of the 22-nt viral siRNAs in both NoV Δ B2 libraries could form canonical siRNA duplexes with 2-nt 3' overhang (Fig. 4B and table S1). The 22-nt viral siRNAs of NoV Δ B2 detected by Northern blotting therefore have the properties of canonical siRNAs processed from dsRNA viral replication intermediates, which demonstrates induction of a typical antiviral RNAi response in mice by NoV Δ B2 infection. Together, our findings reveal that, without viral suppression of RNAi, mice are able to launch a potent antiviral RNAi response sufficiently effective to provide full protection from lethal viral infection.

Here, we have found that an RNA virus infection in cultured hamster cells and suckling mice induces a typical antiviral RNAi response, characterized by the production of viral siRNAs with clearly defined properties of canonical siRNAs. Our findings and those of Maillard *et al.* (28) illustrate that Dicer-dependent processing of

dsRNA viral replication intermediates into successive siRNAs is a conserved mammalian immune response to infection by two distinct positive-strand RNA viruses (table S3). Consistent with the known *in vitro* activity of the B2 protein to inhibit the processing of long dsRNA into siRNAs (3, 16, 21, 24), however, viral small RNAs detected by either deep sequencing or Northern blotting during wild-type NoV infection do not have the properties of canonical siRNAs. Northern blot detection of viral siRNAs in NoV Δ B2-infected mice suggests that the use of *in vivo* infection models and/or viruses incapable of inhibiting siRNA biogenesis may facilitate detection of siRNAs targeting other mammalian viruses. Moreover, NoV infection both *in vitro* and *in vivo* requires the RNAi suppressor activity of its B2 protein. In particular, suckling mice produced abundant viral siRNAs and became completely resistant to the lethal infection by NoV after substitution of a single amino acid in B2 that eliminates its

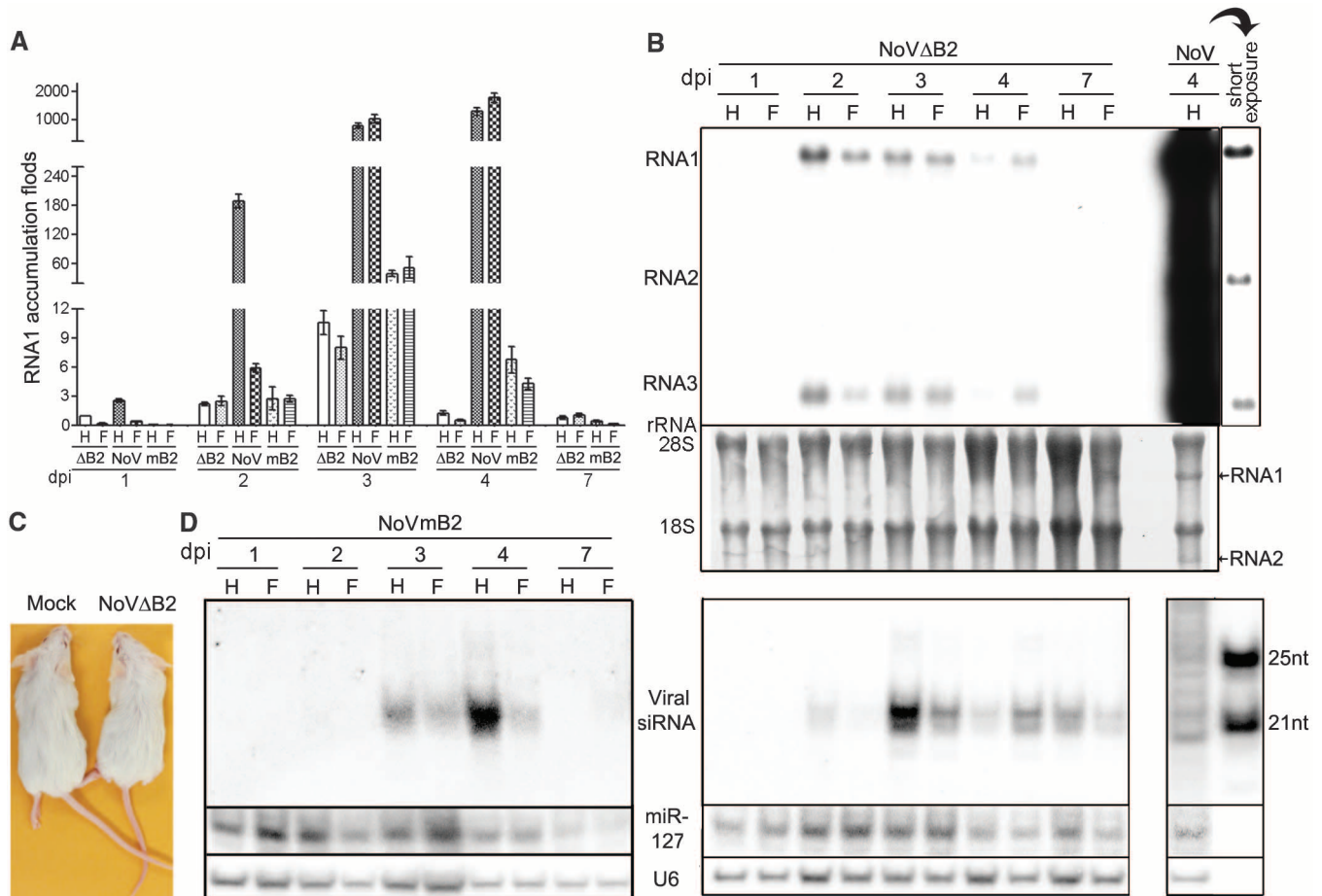


Fig. 3. In vivo virus clearance associated with production of viral siRNAs. (A and B) Accumulation of NoV, NoV Δ B2, and NoVmB2 in mouse fore- (F) and hind- (H) limb tissues detected by quantitative RT-PCR of the viral RNA1 and Northern blotting, respectively. NoV Δ B2 level in hind limb at 1 dpi was set as 1, and error bars indicate standard deviation of three replicates (A). NoV RNAs 1 and 2 (arrows) were visible after rRNA staining to show equal loading (B). (C) Suckling mice remained as healthy 4 weeks post-infection with either NoV Δ B2 (right) or NoVmB2 (not shown) as mock-inoculated

mice (left), whereas all of the five NoV-inoculated mice died by 5 dpi (not shown). (D) Northern blot detection of negative-strand viral siRNAs in mice infected with NoV Δ B2 (middle) or NoVmB2 (left) and of vsRNAs from NoV-infected mice (right). The hybridizing positions of four siRNA probes were given in Fig. 4B, and size markers were synthetic 21- and 25-nt RNAs. The same filters were probed for mouse microRNA 127 (miR-127) and U6 RNA as loading controls. At least three independent repeats with reproducible results were performed with each experiment.

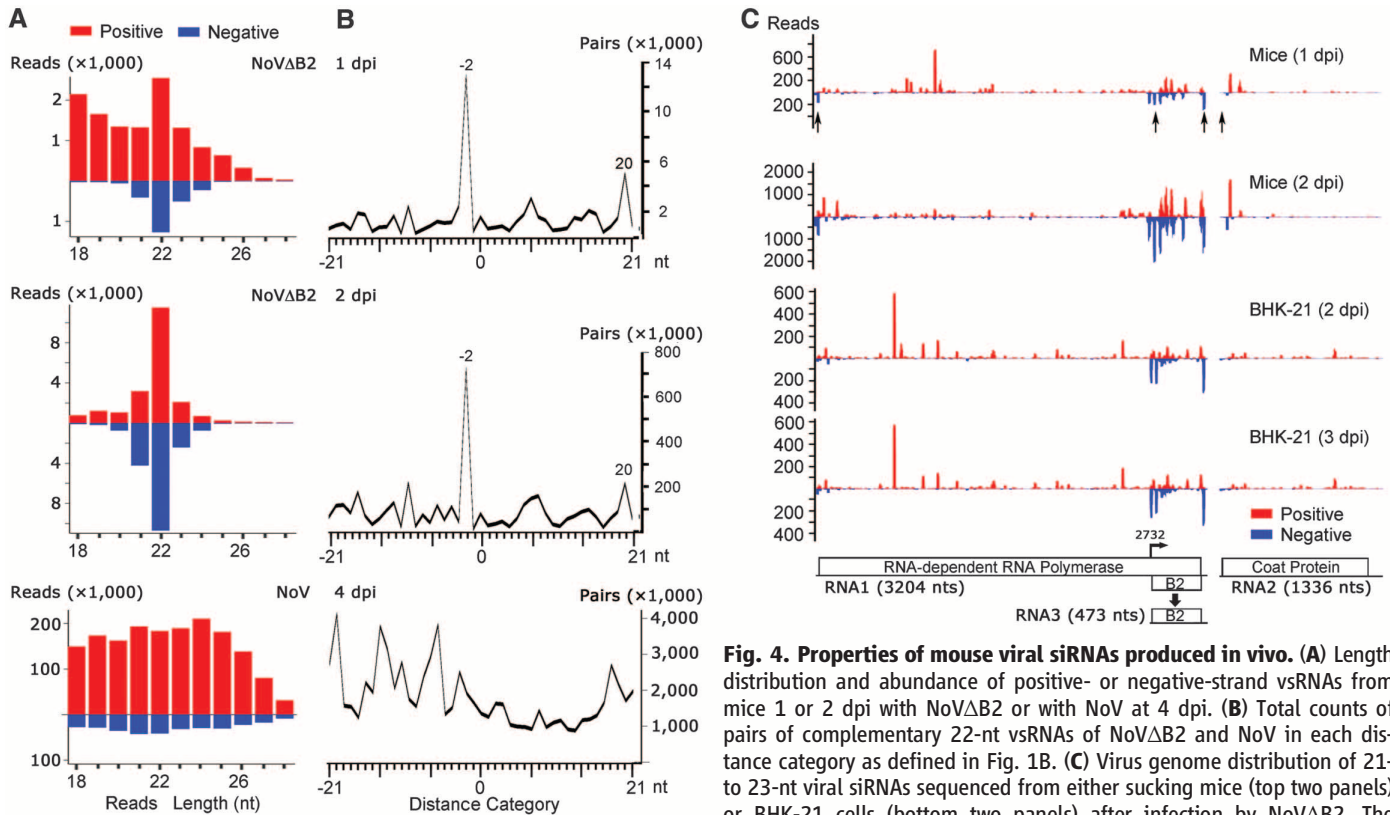


Fig. 4. Properties of mouse viral siRNAs produced in vivo. (A) Length distribution and abundance of positive- or negative-strand vsRNAs from mice 1 or 2 dpi with NoVΔB2 or with NoV at 4 dpi. (B) Total counts of pairs of complementary 22-nt vsRNAs of NoVΔB2 and NoV in each distance category as defined in Fig. 1B. (C) Virus genome distribution of 21- to 23-nt viral siRNAs sequenced from either sucking mice (top two panels) or BHK-21 cells (bottom two panels) after infection by NoVΔB2. The functional proteins encoded by the viral bipartite RNA genome and positions of the four locked nucleic acid probes used to detect negative-

transcription of B2 mRNA (RNA3) from RNA1 are shown. Arrows indicate the positions of the four locked nucleic acid probes used to detect negative-strand viral siRNAs in mice.

RNAi suppressor activity. Thus, the typical RNAi response induced by virus infection in mammals has potent antiviral activity. The striking similarities in the induction and suppression of antiviral RNAi by the closely related FHV and NoV in fruit flies, nematodes, and mammals (2, 3, 8, 12, 21–24) highlight an evolutionary conserved role of RNAi in antiviral defense within the animal kingdom. Compared with the antiviral immunity mechanisms reported to date in mammals, virus clearance by antiviral RNAi has a distinct effector mechanism and does not require cell death (9, 30). Nevertheless, this mammalian immunity mechanism exhibits properties known to be associated with innate and adaptive immunity because it involves rapid host recognition of a microbe-associated molecular pattern dsRNA and a mechanism of specificity determined by pathogen-derived siRNAs (9). Discovery of mammalian antiviral RNAi provides a framework to investigate the innate and adaptive control of important human viral pathogens.

References and Notes

1. A. J. Hamilton, D. C. Baulcombe, *Science* **286**, 950–952 (1999).
2. H. W. Li, W. X. Li, S. W. Ding, *Science* **296**, 1319–1321 (2002).
3. R. Lu et al., *Nature* **436**, 1040–1043 (2005).
4. C. Wilkins et al., *Nature* **436**, 1044–1047 (2005).
5. G. C. Segers, X. Zhang, F. Deng, Q. Sun, D. L. Nuss, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 12902–12906 (2007).
6. I. A. Drinnenberg, G. R. Fink, D. P. Bartel, *Science* **333**, 1592 (2011).
7. M. A. Félix et al., *PLOS Biol.* **9**, e1000586 (2011).
8. X. Guo, W. X. Li, R. Lu, *J. Virol.* **86**, 11645–11653 (2012).
9. S. W. Ding, *Nat. Rev. Immunol.* **10**, 632–644 (2010).
10. A. Deleris et al., *Science* **313**, 68–71 (2006).
11. J. A. Diaz-Pendon, F. Li, W.-X. Li, S.-W. Ding, *Plant Cell* **19**, 2053–2063 (2007).
12. Y. H. Han et al., *J. Virol.* **85**, 13153–13163 (2011).
13. V. Bitko, S. Barik, *BMC Microbiol.* **1**, 34 (2001).
14. P. Parameswaran et al., *PLOS Pathog.* **6**, e1000764 (2010).
15. W. X. Li et al., *Proc. Natl. Acad. Sci. U.S.A.* **101**, 1350–1355 (2004).
16. C. S. Sullivan, D. Ganem, *J. Virol.* **79**, 7371–7379 (2005).
17. Y. Bennasser, S. Y. Le, M. Benkirane, K. T. Jeang, *Immunity* **22**, 607–619 (2005).
18. S. Qian et al., *Proc. Natl. Acad. Sci. U.S.A.* **106**, 605–610 (2009).
19. E. Schnettler et al., *EMBO Rep.* **10**, 258–263 (2009).
20. A. Schneemann, L. A. Ball, C. Delsert, J. E. Johnson, T. Nishizawa, in *Virus Taxonomy—Eighth Report of the International Committee on Taxonomy of Viruses* C. M. Fauquet et al., Eds. (Elsevier, San Diego, CA, 2005), pp. 865–872.
21. J. A. Chao et al., *Nat. Struct. Mol. Biol.* **12**, 952–957 (2005).
22. X. H. Wang et al., *Science* **312**, 452–454 (2006).
23. D. Galiana-Arnoux, C. Dostert, A. Schneemann, J. A. Hoffmann, J. L. Imler, *Nat. Immunol.* **7**, 590–597 (2006).
24. R. Aliyari et al., *Cell Host Microbe* **4**, 387–397 (2008).
25. K. L. Johnson, B. D. Price, L. A. Ball, *Virology* **305**, 436–451 (2003).
26. J. Haasnoot et al., *PLOS Pathog.* **3**, e86 (2007).
27. G. Fabozzi, C. S. Nabel, M. A. Dolan, N. J. Sullivan, *J. Virol.* **85**, 2512–2523 (2011).
28. P. V. Maillard et al., *Science* **342**, 235–238 (2013).
29. L. A. Ball, J. M. Amann, B. K. Garrett, *J. Virol.* **66**, 2326–2334 (1992).
30. D. Goubau, S. Deddouche, C. Reis E Sousa, *Immunity* **38**, 855–869 (2013).

Acknowledgments: We thank J. Jovel, J. Mai, X. Wang, Q. Wu, W.-X. Li, I.-C. Huang, G. Wang, and J. Pedra for technical assistance or advice; K. Johnson, A. Ball, and C. Basler for cDNA clones; and O. Voinnet for comments on the manuscript. X.F. was supported by China Scholarship Council. This study was supported by NIH grants AI52447 and GM94396 and College of Natural and Agricultural Sciences, University of California, Riverside (to S.-W.D.).

Supplementary Materials

www.sciencemag.org/content/342/6155/231/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S3
Tables S1 to S3
References (31–41)

13 June 2013; accepted 14 August 2013
10.1126/science.1241911

Antiviral RNA Interference in Mammalian Cells

P. V. Maillard,^{1*} C. Ciaudo,^{1*} A. Marchais,¹ Y. Li,² F. Jay,¹ S. W. Ding,² Olivier Voinnet^{1†}

In antiviral RNA interference (RNAi), the DICER enzyme processes virus-derived double-stranded RNA (dsRNA) into small interfering RNAs (siRNAs) that guide ARGONAUTE proteins to silence complementary viral RNA. As a counterdefense, viruses deploy viral suppressors of RNAi (VSRs). Well-established in plants and invertebrates, the existence of antiviral RNAi remains unknown in mammals. Here, we show that undifferentiated mouse cells infected with encephalomyocarditis virus (EMCV) or Nodamura virus (NoV) accumulate ~22-nucleotide RNAs with all the signature features of siRNAs. These derive from viral dsRNA replication intermediates, incorporate into AGO2, are eliminated in *Dicer* knockout cells, and decrease in abundance upon cell differentiation. Furthermore, genetically ablating a NoV-encoded VSR that antagonizes DICER during authentic infections reduces NoV accumulation, which is rescued in RNAi-deficient mouse cells. We conclude that antiviral RNAi operates in mammalian cells.

Although mammalian viruses are susceptible to experimental RNA interference (RNAi) via synthetic small interfering RNAs (siRNAs) (1), the existence of a natural antiviral RNAi response in mammals is debated (2). First, in many infected somatic cells, viral double-stranded RNA (dsRNA) triggers the potent and non-sequence-specific interferon (IFN) response (3) that may have largely supplanted antiviral RNAi functions (4). Second, several mammalian viral proteins display viral suppressor of RNAi (VSR)-like activities still awaiting validation in authentic virus expression contexts (1). Third, diverse virus-infected mammalian cell types accumulate virus-derived small RNAs (vsRNAs), but these have unspecified functions (5) and lack the biochemical features, size, and distribution patterns of plant and invertebrate viral siRNAs (6–9). Ascertaining genetically the DICER-dependency of mammalian vsRNA is further complicated by the essential contribution of the mammalian RNAi machinery (one *Dicer*, four *Ago*) to the endogenous microRNA (miRNA) pathway (10). Pluripotent mouse embryonic stem cells (mESCs) withstand the complete ablation of DICER (DCR) or ARGONAUTE (AGO) functions (11, 12) and support RNAi triggered by long dsRNA possibly because they lack an IFN response (13, 14); accordingly, DCR-dependent endogenous siRNAs are detected in these cells (15). We thus reasoned that mESCs constituted potentially valuable models to genetically validate both viral siRNA accumulation and VSR function in authentic mammalian infection contexts.

Several mESC lines were infected with purified virions of encephalomyocarditis virus (EMCV), a mammalian positive-sense single-stranded RNA (ssRNA) picornavirus producing high levels of dsRNA within its 8-hour infection cycle (16). All cells accumulated the EMCV-encoded VP1 capsid to varying degrees, with the highest levels dis-

played by line E14 (Fig. 1A and fig. S1, A and B). In two separate infections, 15- to 50-nt-long RNA was isolated from E14 mESCs and sub-

jected to ILLUMINA deep-sequencing (table S1). Six hours postinfection (hpi), 0.4 and 0.7% of total reads mapped the EMCV genome, of which 33 and 27% were in the 21- to 23-nt size range of DCR products (Fig. 1B and table S1). For comparison, miR-134, miR-296, and miR-470, which functionally target the mESC pluripotency factors *Nanog*, *Oct4*, and *Sox2* (17) represented respectively 0.11%, 0.02%, and 0.05% of total reads (table S2). The remaining EMCV reads, in a heterogeneous 24- to 44-nt size range, mapped nearly exclusively along the viral positive strand (Fig. 1C), which accumulates disproportionately more than the negative strand during positive-sense RNA virus replication, and were thus mostly viral breakdown products (5, 18). By contrast, 36 and 28% of 21- to 23-nt reads mapped to both viral strands within the first 200-nt of the EMCV 5' untranslated region and so exhibited a ~2:1 (+):(-) strand ratio contrasting with the ~10:1 ratio of all other reads (Fig. 1C and table

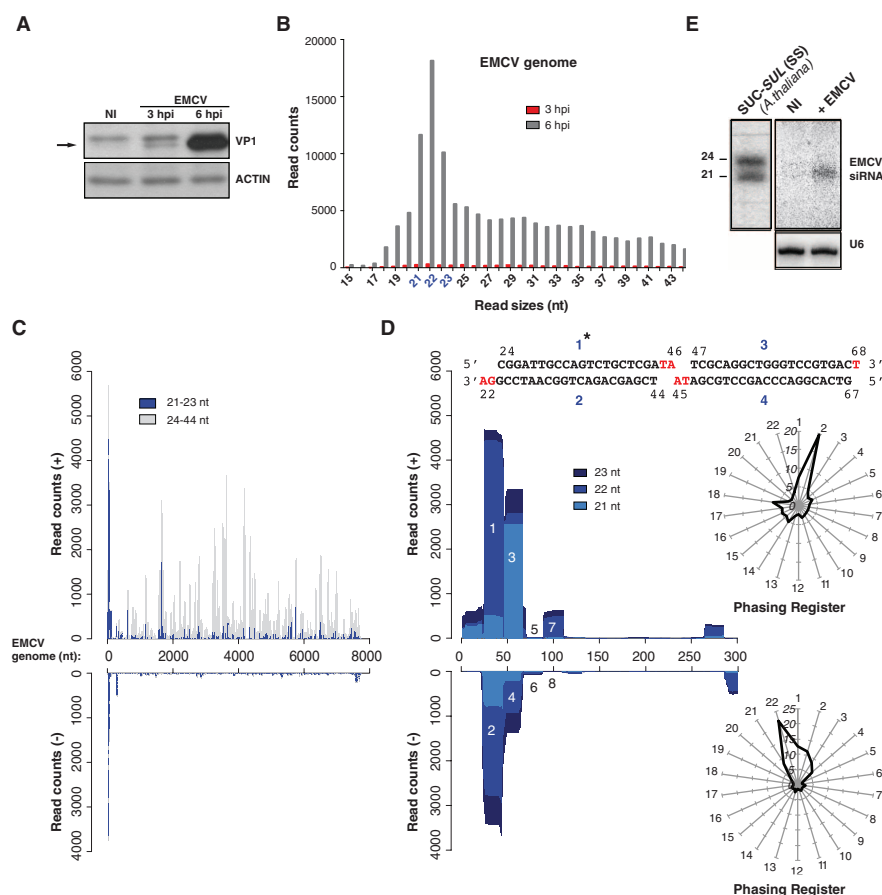


Fig. 1. EMCV-derived siRNAs in infected mESCs. (A) Western analysis of VP1 in E14 mESCs. NI, noninfected; ACTIN, protein-loading control. (B) Size distribution of vsRNA reads mapping to EMCV in samples from (A). (C) Distributions of 21- to 23-nt and 24- to 44-nt reads along EMCV (+) and (-) strands 6 hpi. (D) Same as (C), but along the first 5'-terminal 300 nt. Symmetrical reads are numbered. (Inset) Perfect duplexes formed by the abundant reads 1 to 2 and 3 to 4; 2-nt 3' overhangs are indicated in red. Asterisk: Read sequence corresponding to the oligonucleotide probe used in (E). Radar plots: 21- and 23-nt reads in each of 22 possible registers mapping along the entire EMCV (+) and (-) strands; the 5' first EMCV nucleotide defines register no. 1. Distance to the center indicates read percentage within each register. (E) Northern analysis of EMCV 5'-end siRNAs 6 hpi. Total RNA from SUC-SUL (SS) transgenic *Arabidopsis* run in parallel and hybridized secondarily provides a size marker for 21-nt and 24-nt siRNAs. U6, RNA-loading controls.

¹Department of Biology, Swiss Federal Institute of Technology Zurich (ETH-Z), 8092 Zurich, Switzerland. ²Department of Plant Pathology and Microbiology, University of California, Riverside, CA 92521, USA.

*These authors contributed equally to this work.

†Corresponding author. E-mail: voinneto@ethz.ch

S1). A less-pronounced symmetrical reads distribution was also observed at the EMCV RNA 3'-end, whereas the remaining 21- to 23-nt reads originated from discrete positive-strand regions (Fig. 1C).

The symmetrical 5' and 3' EMCV reads mapped to the regions where dsRNA replication-intermediates (RIs) initiate during positive- and negative-strand synthesis. Similar to RI-derived siRNAs observed in virus-infected plants and invertebrates (6, 9), abundant (+) and (-) reads at the EMCV 5' end formed contiguous and perfectly complementary duplexes with 2-nt 3' overhangs (Fig. 1D). In addition, all EMCV-derived 21- to 23-nt reads defined a dominant, phased register initiated from the 5' end at a ~22-nt periodicity, in which complementary (+) and (-) strands were offset by 2 nt (Fig. 1D and fig. S1, C to E). Northern analyses using oligonucleotide probes confirmed accumulation of the predicted 5'-end 22-nt siRNAs

in EMCV-infected cells (Fig. 1E). Phased, perfect duplexes with 2-nt 3' overhangs are signature products of sequential dicing of long dsRNA (19, 20). The DCR-dependency of EMCV-derived vsRNAs was thus explored in *Dcr* knockout (*Dcr*^{-/-}) mESCs (Fig. 2A and fig. S2A), which, due to pleiotropy and reduced division rates (11), accumulated less EMCV than control *Dcr*^{Flx/Flx} cells. Viral inocula were thus precalibrated to produce comparable infection levels in both cell types (Fig. 2A and fig. S2A). Detected, as expected, in infected *Dcr*^{Flx/Flx} mESCs, EMCV-derived 5'-end vsRNAs were below Northern detection limit in *Dcr*^{-/-} mESCs (Fig. 2A), which confirmed that they were bona fide siRNAs. Moreover, in wild-type (WT) mESCs, these were detected by Northern analysis of RNA from FLAG- and hemagglutinin (HA)-tagged human AGO2 (FLAG-HA-AGO2) immunoprecipitates (IPs) (Fig. 2B and fig. S2B) and by deep-sequencing RNA from

endogenous mAGO2 IPs also containing cellular miRNAs (Fig. 2, C and D, and fig. S2, C and D). Abundant positive-strand reads also coincided with several peaks already observed in total RNA sequencing (Fig. 2C, *), of which one mapped to the internal ribosomal entry site (IRES: nt 577 to 680) and another to a predicted dsRNA fold back (nt 1497 to 1670) (fig. S2E). Therefore, the AGO2-loading landscape of EMCV-infected mESCs comprised RI-derived and DCR-dependent siRNA duplexes, as well as other 21- to 23-nt vsRNAs generated from positive-strand secondary structures via mechanisms that, as in plants and invertebrates, await clarification (6, 9).

The use of mESCs granted an investigation of viral siRNA accumulation in genetically identical cells but under distinct differentiation states. Differentiation of E14-derived embryoid body was confirmed at day 10 by the loss of expression of pluripotency markers *Nanog* and *Oct4* and gain in

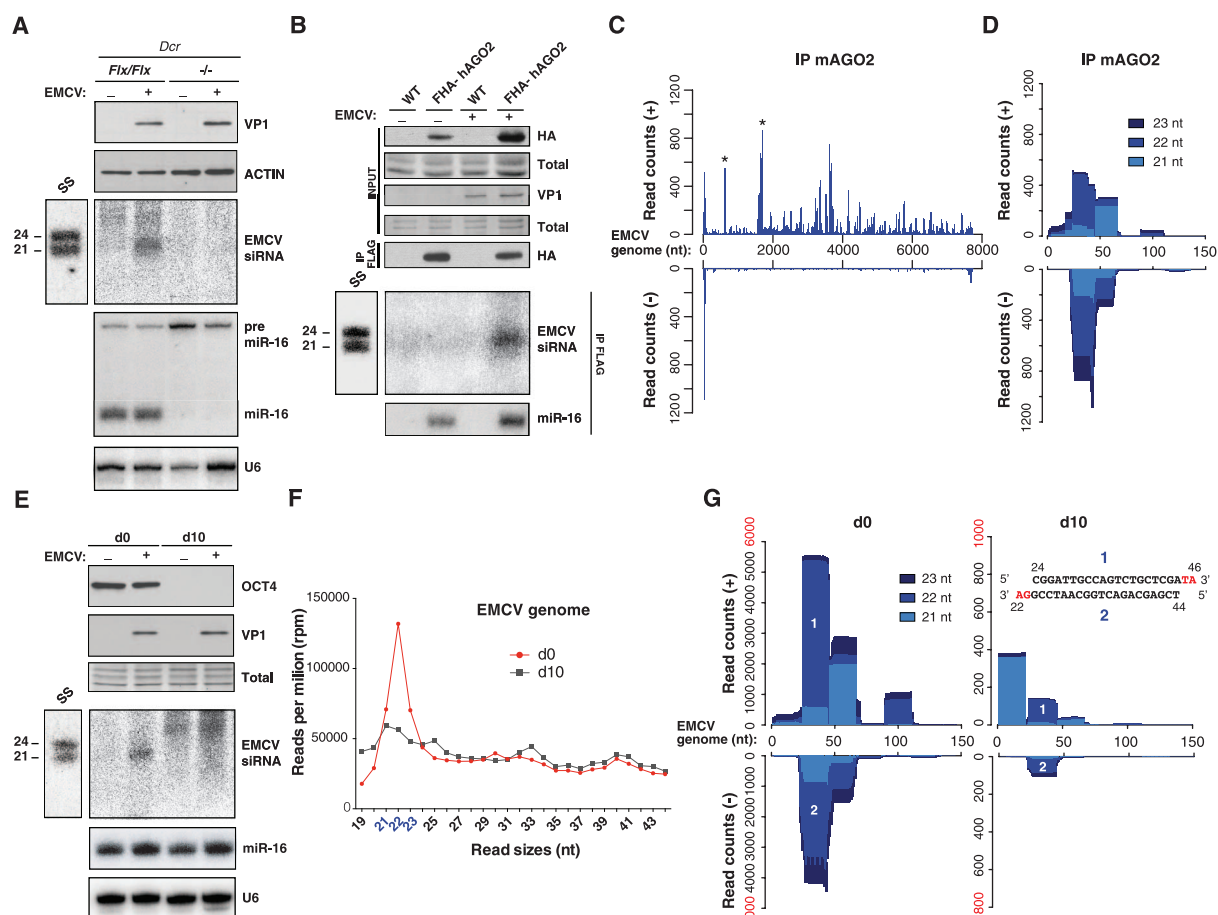


Fig. 2. AGO2-loaded EMCV siRNAs are reduced in *Dcr*^{-/-} mESCs and following differentiation. (A) Western and Northern analysis of VP1 (top), EMCV 5'-end siRNAs (middle) and miR-16/pre-miR-16 (bottom) in *Dcr*^{Flx/Flx} and *Dcr*^{-/-} mESCs infected (+) or not (-) with EMCV. SS, as in Fig. 1E. ACTIN and U6: protein- and RNA-loading controls. (B) Northern analysis of EMCV 5'-end siRNAs 6 hpi in FLAG-specific immunoprecipitates isolated from WT mESCs or mESCs stably expressing human FLAG-HA hAGO2, infected (+) or not (-) with EMCV. SS as in (A). Western analyses show comparable infection levels (VP1) and confirm FHA-hAGO2 immunoprecipitation with miR-16. Total: Coomassie-stained protein loading control. (C) Distributions of 21- and 23-nt reads along EMCV (+) and (-) strands after deep-sequencing of RNA

from endogenous mAGO2 IP 6 hpi. Asterisks: reads further analyzed in fig. S2E. (D) Same as (C), but along the first 5'-terminal 150 nt. (E) Western and Northern analyses of the pluripotency markers OCT4, VP1, EMCV 5'-end siRNAs and miR-16 in undifferentiated mESCs on day 0 (d0) or after 10 days of differentiation (d10), infected (+) or not (-) with EMCV. Total as in (B); U6 and SS are as above. (F) Size distribution of vsRNA deep-sequencing reads mapping to EMCV in samples from (E). Abundance was normalized to the total number of reads mapping to EMCV. (G) Reads mapping to EMCV in infected day 0 and day 10 mESCs, as in Fig. 1D. Note the scale change in counts, highlighted in red. (Inset) siRNA duplex 1 to 2 remains detectable in day 10 cells.

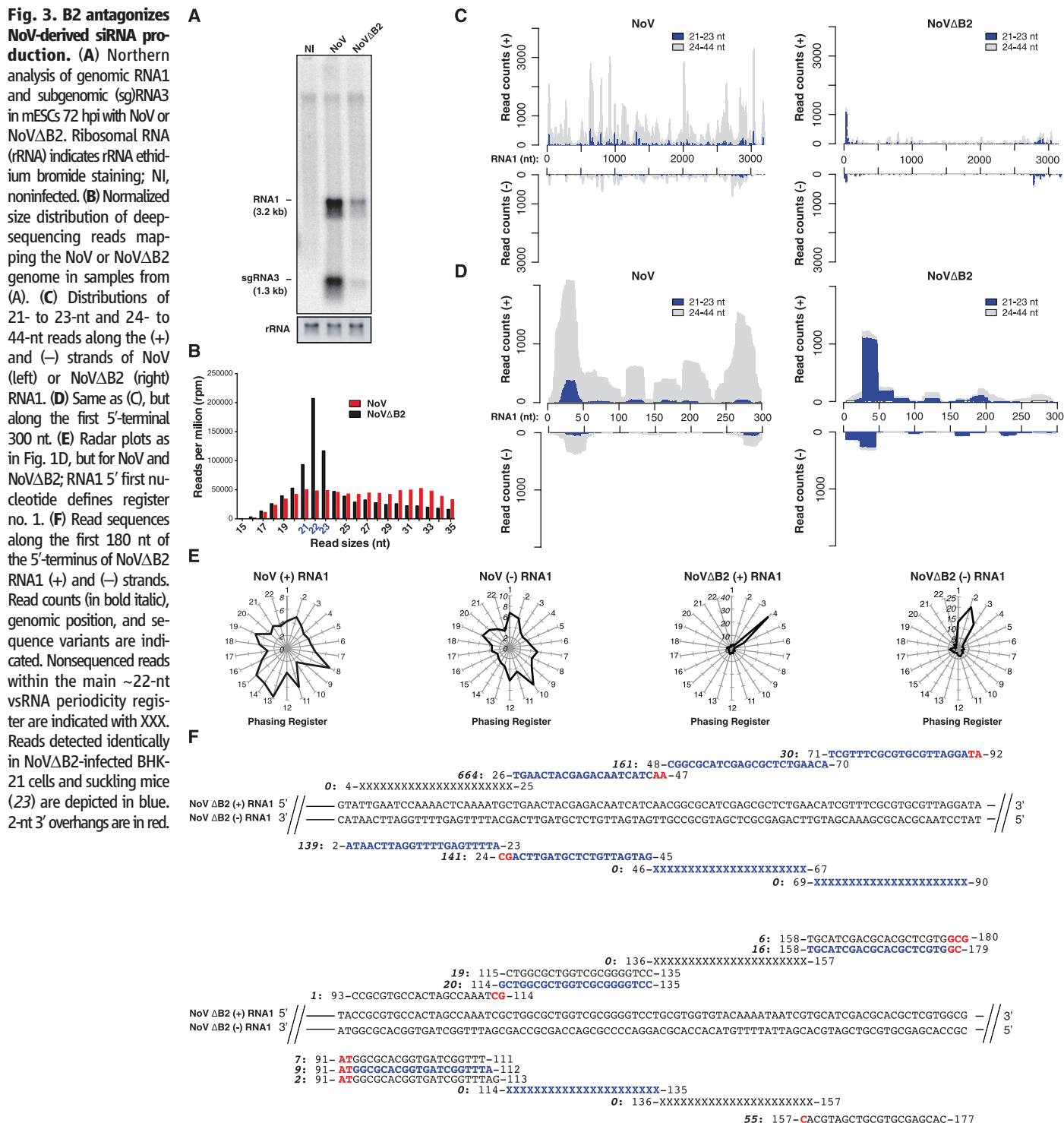
expression of the ectoderm-specific marker *Fgf5* (Fig. 2E and fig. S2F). At 6 hpi, 5'-end siRNAs were below Northern detection in EMCV-infected day 10 compared with day 0 E14 cells, despite their similar infection levels (Fig. 2E and fig. S2F). Accordingly, EMCV-derived reads represented 0.15% of total deep-sequencing reads in infected day 10 cells, a nearly fivefold decrease compared to infected day 0 cells (Fig. 2F). The 21- to 23-nt reads were also 10 times less abundant in day 10 cells

as in day 0 cells but were still detectable, including in the first 5'-terminal 200 nt, representing 16% of all EMCV-derived reads (Fig. 2G and table S1). Therefore, EMCV siRNA accumulation was significantly reduced, albeit not abolished, by differentiation, unlike that of miRNAs (Fig. 2E).

Demonstrating the antiviral activity of siRNAs entails the genetic rescue of VSR-deficient viruses in RNAi-compromised host cells (6, 7, 21), an approach not possible with EMCV for which a

potential VSR awaits identification. The dsRNA-binding B2 protein encoded by the bipartite, positive-sense ssRNA Nodamura virus (NoV) inhibits DCR activity during experimental mammalian RNAi (22), a property shared by its ortholog in the NoV-related Flock house nodavirus (FHV) in *Drosophila* (6). NoV or its B2-deficient counterpart, NoV Δ B2, were titrated to similar levels in stable B2-expressing BHK-21 cells (23) and subsequently used to infect E14 mESCs. NoV Δ B2

Fig. 3. B2 antagonizes NoV-derived siRNA production.



accumulated considerably less than NoV at 3 dpi, and only the former infection was able to generate virus-derived 21- to 23-nt deep-sequencing reads (Fig. 3, A and B, and fig. S3, A and B), whereas global miRNA levels remained unchanged in both infections (tables S1 and S2). NoV-derived reads, heterogeneous in size, mapped mostly along the RNA1 positive strand (Fig. 3C, fig. S3C, and table S1). By contrast, those from NoV Δ B2, nearly exclusively 21 to 23 nt in length, derived mainly from the 5' and 3' ends of RNA1 (+) and (–) strands (Fig. 3, C and D), which resembled the FHV Δ B2 siRNA pattern in *Drosophila* (6). Furthermore, NoV Δ B2 5' end reads had a ~22-nt periodicity and formed contiguous, perfect duplexes with 2- to 3-nt 3' overhangs reminiscent of the DCR-dependent EMCV siRNAs (Fig. 3, E and F). An identical set of phased, perfect duplexes was detected in both NoV Δ B2-infected BHK-21 somatic cells and limbs of newborn mice but not upon

infection with NoV (Fig. 3F) (23). Likewise, reads from the B2-proficient NoV displayed none of these features in mESCs despite their much higher abundance (Fig. 3, E and F, and fig. S3, D and E). Thus, mirroring the action of the FHV-encoded B2 VSR in *Drosophila* (6), DCR-dependent processing of RI-derived dsRNA was suppressed by the NoV-encoded B2 protein in mESCs. Furthermore, this B2-restricted mechanism operated almost identically in mESCs and suckling mice.

FHV B2 inhibits both siRNA processing and incorporation into AGO (24). Therefore, to explore antiviral RNAi in NoV-infected mESCs and to avoid functional redundancy with AGO1, AGO3, or AGO4, we used the quadruple *Ago1,2,3,4* KO mESC line E7, in which an ectopically expressed *hAgo2* transgene is removable by tamoxifen application (12). *hAgo2* depletion was confirmed 5 days after tamoxifen treatment (Fig. 4A), upon which mESCs were infected with NoV or NoV Δ B2 for 3 days. In two separate experiments, the NoV and NoV Δ B2 RNA1 levels were respectively ~2 and ~8 times as high in tamoxifen-induced as in untreated mESCs (Fig. 4B). Northern analyses further showed that NoV Δ B2 accumulation was rescued in AGO2-deficient mESCs similarly to FHV Δ B2 accumulation in *Ago2*-deficient *Drosophila* cells (6, 25); the lower impact of AGO2 depletion on virulent NoV confirmed B2 VSR activity in authentic infections (Fig. 4C). Combined with those obtained with EMCV, the results demonstrate that antiviral RNAi operates in mammalian cells.

The biogenesis and distribution patterns of small RNA derived from ssRNA viruses are thus conserved among infections of plant, invertebrate, and mammalian cells; orthologous VSRs of insect- and mammalian-infecting viruses also suppress DCR action in genetically indistinguishable ways. Therefore, defensive, in addition to possible regulatory, functions likely underpin the evolutionary persistence of catalytic RNAi in mammals. Our results and those of Li *et al.* (23) provide clues as to why mammalian antiviral RNAi has remained elusive thus far. First, previous studies invariably involved virulent viruses, of which some probably encode VSRs that, like the NoV-encoded B2, prevent production of siRNAs, the diagnostic molecules of antiviral RNAi. Second, virus-derived siRNA levels were at least one order of magnitude higher in undifferentiated than in differentiated mESCs or BHK-21 cells (23). This probably relates to the distinctive efficacy of long dsRNA-triggered RNAi in undifferentiated cells derived from embryonic or adult tissues, which is possibly underpinned by their generally reduced ability to mount non-sequence specific immune responses, including the IFN response, against long dsRNA (4). Alternatively, or coincidentally, DCR siRNA-processing activity might decrease during cell differentiation, perhaps via modification of its internal autoinhibitory helicase domain (20). In this context, the identical distribution, relative abundance, and biochemical features of NoV Δ B2 siRNAs observed in mESCs and suckling mice suggest that multipotent pro-

genitor cells, which abound in various mammalian tissues, might form the primary and most potent sites of antiviral RNAi in vivo. Nonetheless, long dsRNA-triggered RNAi was reported in somatic myoblasts, or even in fully differentiated myotubes and neural cells despite the possible activation of an IFN response (13, 26, 27); it would thus be premature to confine the antiviral functions of RNAi to undifferentiated or IFN-deficient cellular states. Tools developed in this and the accompanying study (23) now allow a thorough investigation of these fundamental questions in developing and adult mammals.

References and Notes

1. J. Haasnoot, E. M. Westerhout, B. Berkhout, *Nat. Biotechnol.* **25**, 1435–1443 (2007).
2. J. L. Umbach, B. R. Cullen, *Genes Dev.* **23**, 1151–1164 (2009).
3. D. Goubau, S. Deddouch, C. Reis E Sousa, *Immunity* **38**, 855–869 (2013).
4. K. Chalupnikova, J. Nejezinska, P. Svoboda, *Methods Mol. Biol.* **942**, 291–314 (2013).
5. P. Parameswaran *et al.*, *PLoS Pathog.* **6**, e1000764 (2010).
6. R. Aliyari *et al.*, *Cell Host Microbe* **4**, 387–397 (2008).
7. A. Deleris *et al.*, *Science* **313**, 68–71 (2006).
8. S. W. Ding, O. Voinnet, *Cell* **130**, 413–426 (2007).
9. X. B. Wang *et al.*, *Plant Cell* **23**, 1625–1638 (2011).
10. V. N. Kim, J. Han, M. C. Siomi, *Nat. Rev. Mol. Cell Biol.* **10**, 126–139 (2009).
11. E. P. Murchison, J. F. Partridge, O. H. Tam, S. Cheloufi, G. J. Hannon, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 12135–12140 (2005).
12. H. Su, M. I. Trombly, J. Chen, X. Wang, *Genes Dev.* **23**, 304–317 (2009).
13. P. J. Paddison, A. A. Caudy, G. J. Hannon, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 1443–1448 (2002).
14. E. Billy, V. Brondani, H. D. Zhang, U. Müller, W. Filipowicz, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 14428–14433 (2001).
15. J. E. Babiarz, J. G. Ruby, Y. Wang, D. P. Bartel, R. Blelloch, *Genes Dev.* **22**, 2773–2785 (2008).
16. F. Weber, V. Wagner, S. B. Rasmussen, R. Hartmann, S. R. Paludan, *J. Virol.* **80**, 5059–5064 (2006).
17. Y. Tay, J. Zhang, A. M. Thomson, B. Lim, I. Rigoutsos, *Nature* **455**, 1124–1128 (2008).
18. S. Pfeffer *et al.*, *Nat. Methods* **2**, 269–276 (2005).
19. E. Allen, Z. Xie, A. M. Gustafson, J. C. Carrington, *Cell* **121**, 207–221 (2005).
20. E. Ma, I. J. MacRae, J. F. Kirsch, J. A. Doudna, *J. Mol. Biol.* **380**, 237–243 (2008).
21. J. A. Diaz-Pendon, F. Li, W. X. Li, S. W. Ding, *Plant Cell* **19**, 2053–2063 (2007).
22. C. S. Sullivan, D. Ganem, *J. Virol.* **79**, 7371–7379 (2005).
23. Y. Li, J. Lu, Y. Han, X. Fan, S.-W. Ding, *Science* **342**, 231–234 (2013).
24. J. A. Chao *et al.*, *Nat. Struct. Mol. Biol.* **12**, 952–957 (2005).
25. X. H. Wang *et al.*, *Science* **312**, 452–454 (2006).
26. C. E. Yi, J. M. Bekker, G. Miller, K. L. Hill, R. H. Crosbie, *J. Biol. Chem.* **278**, 934–939 (2003).
27. L. Gan *et al.*, *J. Neurosci. Methods* **121**, 151–157 (2002).

Acknowledgments: We thank the Voinnet laboratory for advice and discussions and L. Bakali-Kassimi, G. Meister, E. Brocchi, and J.-C. Paillart for reagents. Supported by a Prospective Researchers Fellowship from the Swiss National Foundation to P.V.M., a fellowship from the Federation of European Biochemical Societies to C.C., and an award from the Bettencourt Foundation to O.V.

Supplementary Materials

www.sciencemag.org/content/342/6155/235/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S3
Tables S1 to S3
References (28–48)

14 June 2013; accepted 14 August 2013
10.1126/science.1241930

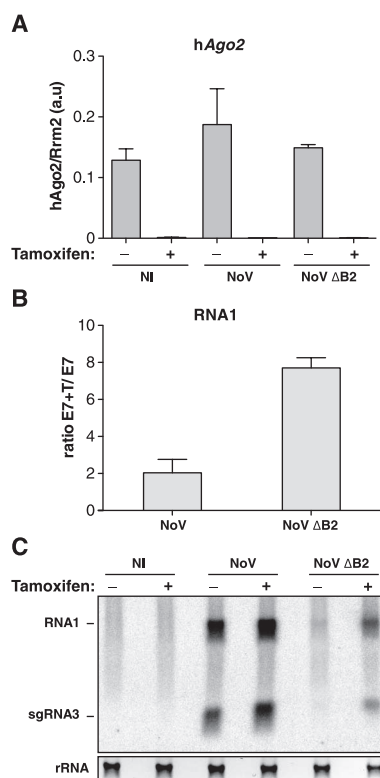


Fig. 4. Rescue of NoV Δ B2 accumulation in AGO2-deficient mESCs. (A) Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of the *hAgo2* transgene mRNA levels in noninfected (NI) and NoV- and NoV Δ B2-infected E7 mESCs previously treated (+) or not (–) with tamoxifen for 5 days. Results show the mean and standard deviation of two independent experiments; a.u., arbitrary units. (B) Relative accumulation of NoV or NoV Δ B2 RNA1 72 hpi in E7 mESC treated (+T) or not with tamoxifen, assessed by quantitative RT-PCR on samples used in (A). Results show the mean of the ratio and the standard deviation calculated from two independent experiments. (C) Northern analysis of NoV and NoV Δ B2 genomic RNA1 and sgRNA3 72 hpi of the cells used in (A). rRNA, as in Fig. 3A.

RTEL1 Is a Replisome-Associated Helicase That Promotes Telomere and Genome-Wide Replication

Jean-Baptiste Vannier,^{1*} Sumit Sandhu,^{2*} Mark IR. Petalcorin,¹ Xiaoli Wu,² Zinnatun Nabi,² Hao Ding,^{2,3†} Simon J. Boulton^{1†}

Regulator of telomere length 1 (RTEL1) is an essential DNA helicase that disassembles telomere loops (T loops) and suppresses telomere fragility to maintain the integrity of chromosome ends. We established that RTEL1 also associates with the replisome through binding to proliferating cell nuclear antigen (PCNA). Mouse cells disrupted for the RTEL1-PCNA interaction (PIP mutant) exhibited accelerated senescence, replication fork instability, reduced replication fork extension rates, and increased origin usage. Although T-loop disassembly at telomeres was unaffected in the mutant cells, telomere replication was compromised, leading to fragile sites at telomeres. RTEL1-PIP mutant mice were viable, but loss of the RTEL1-PCNA interaction accelerated the onset of tumorigenesis in p53-deficient mice. We propose that RTEL1 plays a critical role in both telomere and genome-wide replication, which is crucial for genetic stability and tumor avoidance.

The stability of the genome is critically dependent on the coordinate action of DNA repair pathways during the cell cycle (1). The DNA helicase regulator of telomere length 1 (RTEL1) is an anti-recombinase that dismantles D-loop recombination intermediates to counter

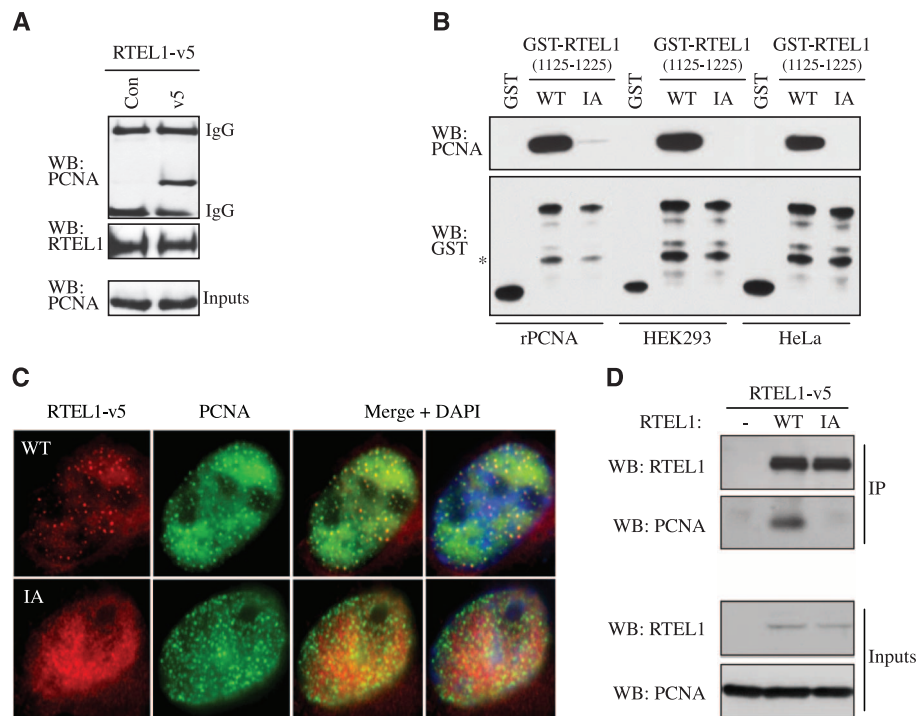
toxic DNA repair (2). RTEL1 also functions in meiosis to limit excessive crossing over (3) and disassembles T loops and suppresses telomere fragility to maintain integrity of the chromosome end (4). The mechanism(s) by which RTEL1 executes its function at sites of DNA repair and at telomeres remains unclear. Because *RTEL1*^{−/−} mice are embryonic lethal (5) and die mid-gestation, it is also possible that RTEL1 possesses other essential functions that remain to be defined.

To further investigate RTEL1 function in cells, we performed mass spectrometry to identify interacting proteins from stable cells expressing a C-terminal green fluorescent protein (GFP)-tagged

RTEL1. In addition to methyl methanesulfonate sensitive 19 (MMS19) that functions in Fe-S cluster assembly (6), we detected numerous components of the replisome, including replication factor C (RFC), DNA polymerases, minichromosome maintenance proteins (MCMs), and proliferating cell nuclear antigen (PCNA) (fig. S1A). We further investigated a putative RTEL1-PCNA interaction as bioinformatic analysis revealed the presence of a putative PCNA interaction motif (PIP box) located in the C terminus of the RTEL1 protein (fig. S1B). RTEL1-v5 and endogenous PCNA coimmunoprecipitated from cell extracts, and this interaction was resistant to benzonase treatment, excluding a nonspecific association via DNA bridging (Fig. 1A). By contrast, RTEL1-v5 proteins harboring five PIP box substitution mutations failed to immunoprecipitate PCNA (fig. S1C). The RTEL1-PCNA interaction is likely direct as in vitro pull-down experiments with a glutathione *S*-transferase (GST) fusion to a fragment of RTEL1 (amino acids 1125 to 1225) containing the wild-type PIP box efficiently bound to recombinant PCNA (rPCNA) as well as endogenous PCNA from human embryonic kidney-293 (HEK293) or HeLa cell extracts, whereas a GST-RTEL1 fusion harboring an I1169A PIP box mutant (referred to as IA) failed to interact (Fig. 1B). Arrayed biotinylated peptides comprising the wild-type RTEL1 PIP box sequence bound to rPCNA, whereas the RTEL1 PIP box mutant peptides did not (fig. S1D). These results demonstrate that the PIP box in RTEL1 confers a direct interaction with PCNA in vitro and in cells.

PCNA is a processivity factor for DNA polymerase and an integral component of the replisome during S phase. PCNA is present in multiple

Fig. 1. RTEL1-PCNA interaction is dependent on RTEL1 PIP motif. (A) Immunoprecipitation of RTEL1-v5 in ES cells stably overexpressing RTEL1-v5. IgG, immunoglobulin G; WB, Western blot. **(B)** GST pull-down experiments between wild-type (WT) RTEL1 or IA RTEL1 mutant and recombinant PCNA (rPCNA) extracts from HEK293 or from HeLa cells. (*) Truncated protein. **(C)** Detection of RTEL1-v5 (red) and PCNA (green) in ES cells overexpressing WT or IA RTEL1-v5 and PCNA-flag. DAPI, 4',6-diamidino-2-phenylindole. **(D)** Immunoprecipitation (IP) of RTEL1-v5 and RTEL1-IA-v5 from ES cells derived from *RTEL1*^{+/+}v5 and *RTEL1*^{IA/IA}v5 mice.



discrete replication foci in S-phase cells (Fig. 1C) (7). Wild-type RTEL1-v5 also formed discrete foci, a subset of which colocalized with PCNA-Flag in unperturbed cells (Fig. 1C). The RTEL1 IA PIP box mutant is expressed in the nucleus, but failed to colocalize with PCNA (Fig. 1C). Thus, the presence of RTEL1 within replication foci is dependent on a PIP box-mediated interaction with PCNA. This result also suggests that any additional contacts between RTEL1 and other replisome components are likely to be weak or indirect, as they are not sufficient to maintain RTEL1 at replication foci when the RTEL1-PCNA interaction is abolished.

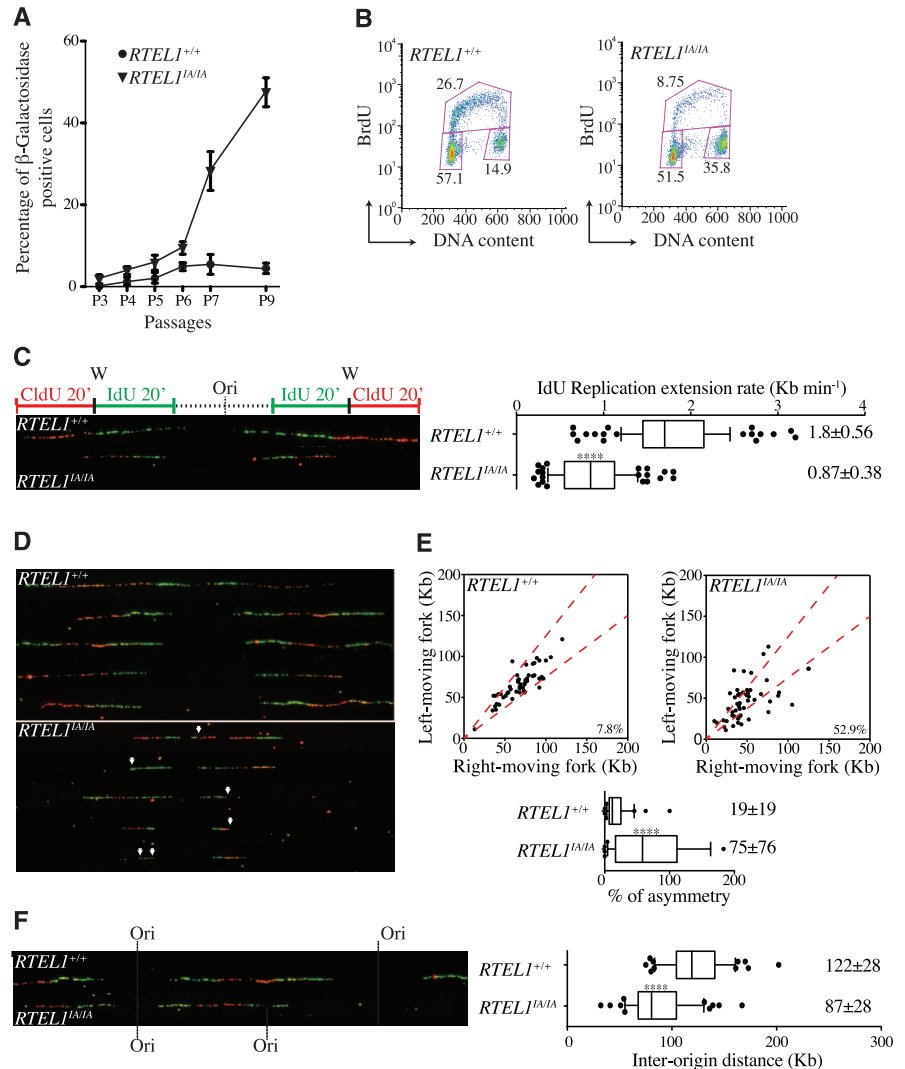
To investigate the functional importance of the RTEL1-PCNA interaction in vivo, RTEL1 IA PIP mutation marked with a v5 epitope was knocked into the *RTEL1* locus in mice (fig. S2). The resultant mouse allele, termed *RTEL1*^{IA/v5}, abolished RTEL1-PCNA binding as compared to the control mouse allele (*RTEL1*^{+/+}v5), which retains an intact RTEL1 PIP motif (Fig. 1D). Loss of the RTEL1-PCNA interaction induced growth arrest and senescence in primary mouse embryonic fibroblasts (MEFs) derived from em-

bryonic day 14.5 (E14.5) *RTEL1*^{IA/v5} embryos when compared to *RTEL1*^{+/+}v5 control MEFs (Fig. 2A and fig. S3A), which we attribute to increased levels of spontaneous DNA damage in the mutant cells (fig. S5, E and F). Cell cycle analysis revealed a significant reduction in 5-bromo-2'-deoxyuridine (BrdU) incorporation and an accumulation of cells in late S/G₂ in primary *RTEL1*^{IA/v5} MEFs when compared to control, suggestive of a defect in DNA replication (Fig. 2B and fig. S3B). Indeed, measurement of the progression of sister replication forks by molecular combing revealed that loss of the RTEL1-PCNA interaction caused forks to move considerably more slowly in a genome-wide manner compared to control cells (0.87 and 1.8 kb min⁻¹, respectively; Fig. 2C). Of the replication forks, 52.9% were highly asymmetrical in *RTEL1*^{IA/v5} cells, compared to 7.8% asymmetrical tracts in *RTEL1*^{+/+}v5 control cells (Fig. 2, D and E), which suggests that loss of the RTEL1-PCNA interaction in cells results in increased replication fork stalling and/or collapse. Interorigin distances were also significantly shorter in the *RTEL1*^{IA/v5} cells when compared to control cells (87 ± 28 and 122 ± 28 kb,

respectively; Fig. 2F), indicative of increased origin firing in mutant cells. Similar replication defects were also observed after conditional inactivation of RTEL1 following adenovirus-Cre treatment of *RTEL1*^{F/F} MEFs (fig. S3, C to F). Blocking replication origin firing in the *RTEL1*^{IA/v5} cells with the CDC7 inhibitor PHA-767491 (fig. S4A) (8, 9) rescued both interorigin distance and replication fork extension rates to wild type but failed to rescue fork asymmetry (fig. S4, B to D). This suggests that increased origin usage and the reduction in fork speeds in *RTEL1*^{IA/v5} cells occur as a secondary consequence of a defect in preventing fork stalling and/or collapse. We propose that in the absence of the RTEL1-PCNA interaction, increased fork stalling and/or collapse or a failure to repair and/or restart a subset of replication forks triggers dormant origin firing, which in turn leads to a global reduction in replication fork extension rates.

RTEL1 is proposed to maintain telomere integrity in part by catalyzing T-loop disassembly during S phase (4). Failure to dismantle T loops after inactivation of *RTEL1* is associated with rapid telomere shortening and loss of the T loop

Fig. 2. RTEL1-PCNA interaction promotes normal genome replication. (A) Quantification of growth cell arrest after consecutive passages (P1 to P9) of each genotype. (B) BrdU incorporation of *RTEL1*^{IA/v5} and *RTEL1*^{+/+}v5 primary MEFs. (C) Replication fork dynamics in *RTEL1*^{IA/v5} and control MEFs pulse-labeled with iododeoxyuridine (IdU) or chlorodeoxyuridine (CldU) and subjected to DNA combing. One hundred fibers were measured per genotype, and replication fork speed was measured in kb min⁻¹. (D) Representative images of sister replication forks in *RTEL1*^{+/+}v5 and *RTEL1*^{IA/v5} primary MEFs. Arrows mark unstable and/or stalled forks. (E) Quantification of fork asymmetry in *RTEL1*^{IA/v5} and control primary MEFs. (F) Representative images and quantification of inter-origin distances (kb) in *RTEL1*^{IA/v5} and *RTEL1*^{+/+}v5. Fifty fibers were measured per genotype. *****P* < 0.0001 (two-tailed Mann-Whitney test). Error bars show the SD.



as a circle (4). Phi29 polymerase-dependent telomere circles accumulate in cells after conditional inactivation of *RTEL1* in *RTEL1^{F/F}* MEFs (Fig. 3A) (4). By contrast, telomere circles (TCs) were undetectable in either *RTEL1^{+/+}*v5- or *RTEL1^{IA/IA}*v5-complemented embryonic stem (ES) cells (Fig. 3A). We also found no evidence of telomere loss in metaphase spreads of *RTEL1^{+/+}*v5 or *RTEL1^{IA/IA}*v5 cells (Fig. 3, B and C). *RTEL1^{+/+}*v5 or *RTEL1^{IA/IA}*v5 cells also exhibited a wild-type telomere length distribution (fig. S5A) and did not show any evidence of DNA damage at telomeres (fig. S5B), increased sister chromatid exchanges (SCEs), or increased telomere SCEs (fig. S5, C and D). Because *RTEL1^{IA/IA}*v5 cells do not exhibit a significant induction of DNA damage at telomeres (fig. S5B), the elevated spontaneous levels of the histone γ H2AX in these cells (fig. S5, E and F) are not caused by dysfunctional telomeres. These results establish that the RTEL1-PCNA interaction is dispensable for T-loop disassembly and preventing loss of the telomere as a circle.

RTEL1 is also responsible for the suppression of telomere fragility (4, 10). Loss of *RTEL1* in ES cells resulted in enhanced telomere fragility (24 ± 10 fragile telomeres per metaphase, compared to 8.8 ± 3.6 and 9.9 ± 4.2 in wild-type ES cells and *RTEL1^{-/-}* ES cells complemented with *RTEL1^{+/+}*v5; Fig. 3B and table S1). *RTEL1^{-/-}* ES cells complemented with *RTEL1^{IA}*v5 PIP box mutant and *RTEL1^{IA/IA}*v5 MEFs exhibited high

levels of telomere fragility (20 ± 7.9 and 10 ± 4.9 , respectively; Fig. 3, B and C, and table S1), comparable to those detected in *RTEL1*-null ES cells or *RTEL1^{-/-}* MEFs [24 ± 10 and (4), respectively]. After conditional inactivation of *RTEL1* in *RTEL1^{F/F}* MEFs, telomere fragility is exacerbated by treatment with aphidicolin or the G4-DNA stabilizer TMPyP4 (4). Similarly, treatment of *RTEL1^{IA/IA}*v5 MEFs with aphidicolin or TMPyP4 resulted in a significant increase in telomere fragility, corresponding to 15 ± 5.5 and 14 ± 6.2 fragile telomeres per metaphase compared to 10 ± 4.9 fragile telomeres per metaphase without treatment (Fig. 3C and table S1). Collectively, these results establish that the RTEL1-PCNA interaction is essential for suppressing telomere fragility but is dispensable for T-loop disassembly (fig. S5G).

RTEL1-deficient cells are sensitive to G4-DNA stabilizer TMPyP4 (11) and telomere fragility is exacerbated by telomeric G4-DNA stabilization (4), suggesting a strong correlation between fragile telomeres and G4-DNA secondary structures. To address whether RTEL1 can disassemble telomeric G4-DNA, recombinant RTEL1 wild-type and adenosine triphosphatase (ATPase) dead (RTEL1 K48R) mutant proteins were purified and tested for activity toward telomeric G4-DNA structures. RTEL1 wild-type efficiently disassembled telomeric G4-DNA structures in an ATPase-dependent manner in vitro (fig. S6A). Addition of recombinant PCNA to the reaction neither stimulated nor inhibited the re-

action. However, addition of TMPyP4 to the reaction inhibited the ability of RTEL1 wild type to disassemble the substrate (fig. S6B), suggesting that TMPyP4 exacerbates telomere fragility in vivo by blocking the ability of RTEL1 to dismantle telomeric G4-DNA structures.

RTEL1 was recently implicated in Hoyeraal-Hreidarsson syndrome, a severe form of the cancer predisposition and bone marrow failure syndrome dyskeratosis congenita (12–14). A genome-wide association study also identified RTEL1 as a susceptibility locus for glioma (15, 16), raising the possibility that RTEL1 may function as a tumor suppressor. Despite the genome-wide replication problems in primary cells, *RTEL1^{IA/IA}*v5 mutant mice are born at expected Mendelian ratios and exhibit normal body weight and morphology at adult stage. The viability of these mice is potentially explained by increased origin usage that likely compensates for the replication defects in cells. To examine the role of the RTEL1-PCNA interaction during tumor development, we established a cohort of compound *Trp53^{-/-}* *RTEL1^{IA/IA}*v5 and *Trp53^{-/-}* *RTEL1^{IA/IA}*v5 mutant mice and monitored their survival. Homozygous *Trp53^{-/-}* *RTEL1^{IA/IA}*v5 mice exhibited substantially shorter life spans compared with control *Trp53^{-/-}* *RTEL1^{+/+}*v5 mice ($P < 0.0002$; Fig. 4A). Tumor formation occurred in the *Trp53^{-/-}* *RTEL1^{IA/IA}*v5 mice within 115 ± 31 days compared with 147 ± 35 days for

Fig. 3. RTEL1-PCNA interaction is required to suppress telomere fragility but is dispensable to T-loop disassembly. (A) Phi29-dependent TCs amplification and quantification in *RTEL1^{+/+}*, *RTEL1^{-/-}*, and ES cells complemented with *RTEL1^{+/+}*v5 (+/+) and *RTEL1^{IA}*-v5 (IA/IA). (B) Quantification of telomere loss and fragile telomeres per metaphase in *RTEL1^{+/+}*, *RTEL1^{-/-}*, and ES cells complemented (Comp.) with *RTEL1^{+/+}*v5 (Wt) and *RTEL1^{IA}*-v5 (IA/IA). (C) Representative images and quantification of fragile telomeres (arrow) and telomere loss (*) per metaphase in *RTEL1^{+/+}*v5 and *RTEL1^{IA/IA}*v5 primary MEFs subjected to the indicated treatments (–, no treatment; APD, aphidicolin; statistical analysis with two-tailed Mann-Whitney test). Error bars show the SD.

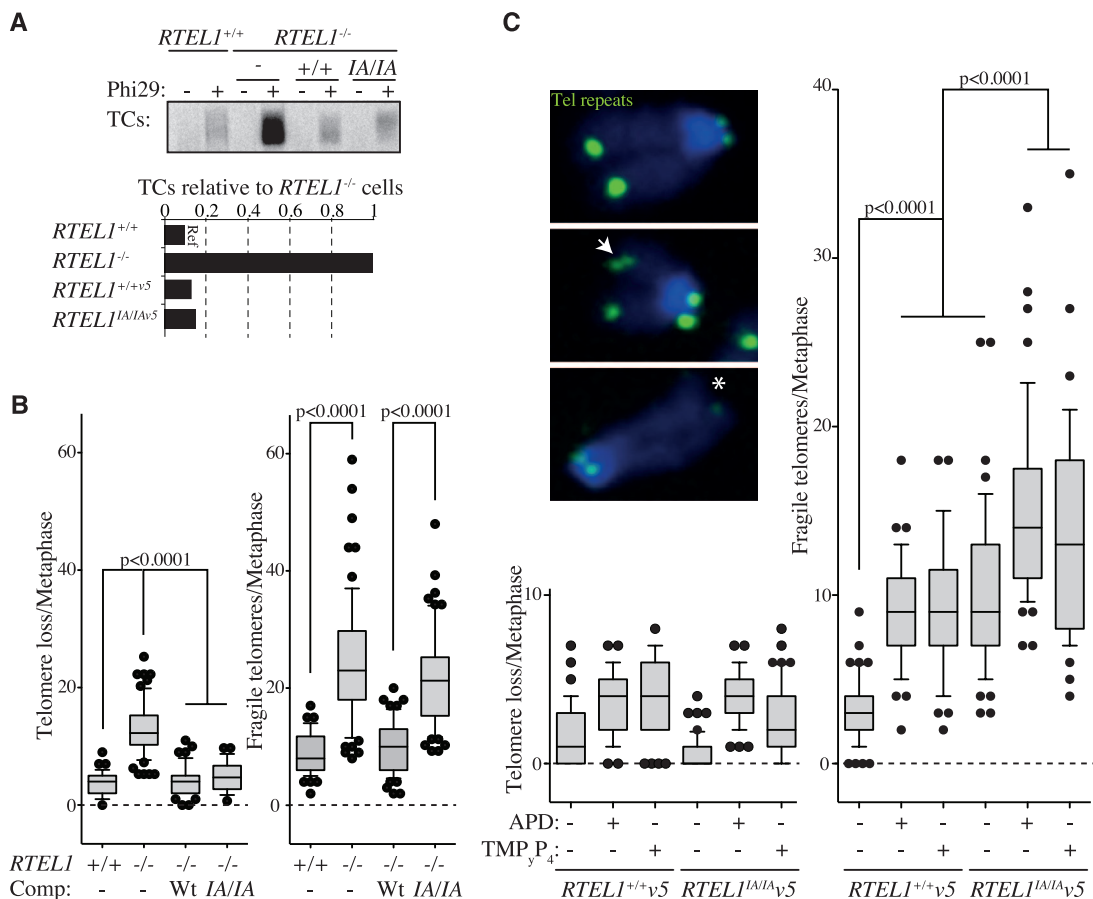
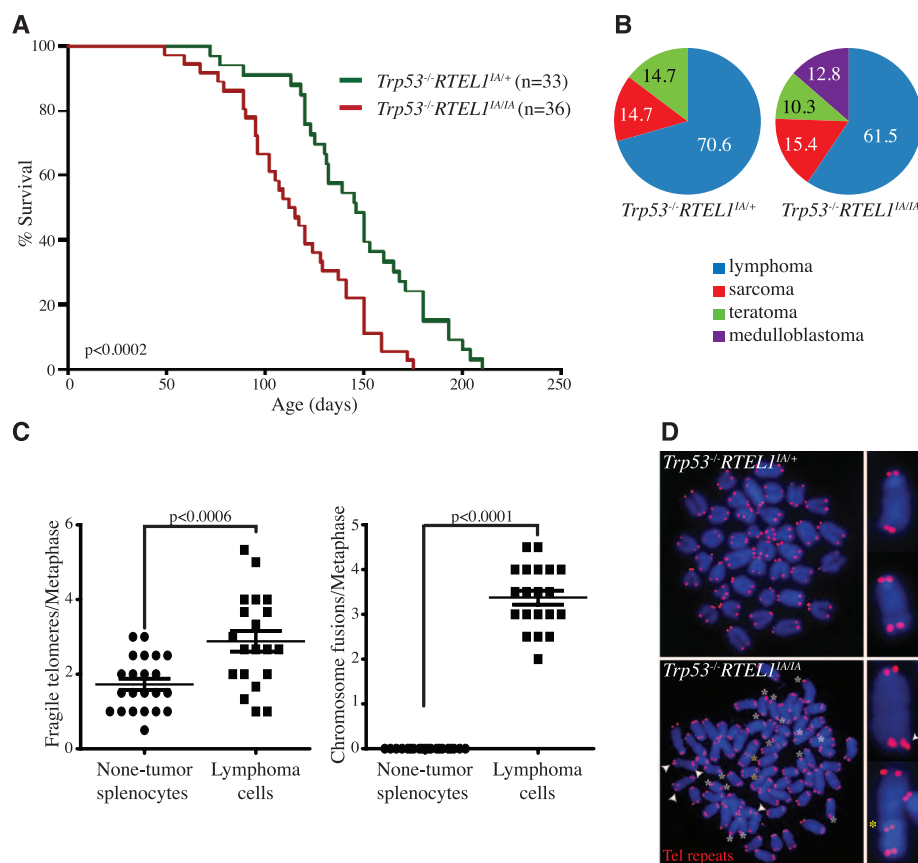


Fig. 4. RTEL1^{IA/IA} mutation accelerates tumorigenesis in mice. (A) Kaplan-Meier survival curves of *Trp53*^{-/-}*RTEL1*^{IA/+}*v5* and *Trp53*^{-/-}*RTEL1*^{IA/IA}*v5* mice with the number of mice indicated for each genotype. (B) Tumor spectrum in *RTEL1*^{IA/+}*v5* and *RTEL1*^{IA/IA}*v5* mice in *Trp53*^{-/-} background. (C) Frequency of fragile telomeres and chromosome fusions in *Trp53*^{-/-}*RTEL1*^{IA/IA}*v5* tumorigenic and nontumorigenic tissues. *P* value was calculated with two-tailed Mann-Whitney test. (D) Representative images of metaphase spreads derived from *Trp53*^{-/-}*RTEL1*^{IA/+}*v5* and *Trp53*^{-/-}*RTEL1*^{IA/IA}*v5* tumors subjected to telomere fluorescence in situ hybridization (red, telomeres; blue, DNA). White asterisk (*), sister chromatid fusions; yellow asterisk, end-to-end fusions; arrows, fragile telomeres.



Trp53^{-/-}*RTEL1*^{IA/IA}*v5* mice. Histological analysis showed that both cohorts of *Trp53*^{-/-}*RTEL1*^{IA/+}*v5* and *Trp53*^{-/-}*RTEL1*^{IA/IA}*v5* mutant mice developed predominantly lymphomas (61.5 and 70.6%, respectively; Fig. 4B), although sarcomas and teratomas were also observed (15.4 versus 14.7% and 10.3 versus 14.7%, respectively; Fig. 4B), as described previously in *Trp53*^{-/-} mice (17). Telomere analysis of the tumors from *Trp53*^{-/-}*RTEL1*^{IA/IA}*v5* mice revealed extensive sister chromatid fusions, end-to-end fusions with telomeric repeats, and a high frequency of fragile telomeres when compared to nontumor cells from the same mice (Fig. 4, C and D, and fig. S7I). Furthermore, tumors from *Trp53*^{-/-}*RTEL1*^{IA/+}*v5* mice or *Trp53*^{-/-}*RTEL1*^{IA/IA}*v5* mice exhibited no evidence of telomere fragility or dysfunction (Fig. 4, C and D). *Trp53*^{-/-}*RTEL1*^{IA/IA}*v5* mutant mice also developed medulloblastomas (12.8%; Fig. 4B and fig. S7, A to F), which were not observed in the *Trp53*^{-/-}*RTEL1*^{IA/+}*v5* mice or in *Trp53*^{-/-} mice described previously (17). Because RTEL1 is expressed in the cerebellar external granular layer (EGL) cells (fig. S7, G and H), which are the source of medulloblastomas (18), these data suggest that the RTEL1-PCNA interaction is important for protecting these EGL cells from transformation.

Our data reveal a critical interaction between RTEL1 and PCNA that is essential for telomere and genome-wide replication and suppression of tumorigenesis in vivo. Suppression of telomere fragility, which we attribute to the telomeric G4-

DNA unwinding activity of RTEL1, is absolutely dependent on the RTEL1-PCNA interaction. This suggests that replisome association is required for RTEL1 to counteract telomeric G4-DNA structures that arise at the replication fork. By contrast, the role for RTEL1 in T-loop disassembly and the suppression of telomere loss as a circle does not require the RTEL1-PCNA interaction. The RTEL1-PCNA interaction is also necessary to prevent replication fork stalling and/or collapse, which affects genome-wide replication. Finally, the accelerated rate of tumor formation and predisposition to medulloblastomas conferred by the *RTEL1*^{IA/IA}*v5* mutation in the *Trp53*^{-/-} background implicates RTEL1 as a tumor-suppressor gene.

References and Notes

- J. R. Chapman, M. R. Taylor, S. J. Boulton, *Mol. Cell* **47**, 497–510 (2012).
- L. J. Barber *et al.*, *Cell* **135**, 261–271 (2008).
- J. L. Youds *et al.*, *Science* **327**, 1254–1258 (2010).
- J. B. Vannier, V. Pavicic-Kaltenbrunner, M. I. Petalcorin, H. Ding, S. J. Boulton, *Cell* **149**, 795–806 (2012).
- H. Ding *et al.*, *Cell* **117**, 873–886 (2004).
- K. Gari *et al.*, *Science* **337**, 243–245 (2012).
- J. Essers *et al.*, *Mol. Cell. Biol.* **25**, 9350–9359 (2005).
- A. Montagnoli *et al.*, *Nat. Chem. Biol.* **4**, 357–365 (2008).
- L. C. Chuang *et al.*, *Mol. Cell* **35**, 206–216 (2009).
- A. Sfeir *et al.*, *Cell* **138**, 90–103 (2009).
- E. J. Uringa *et al.*, *Mol. Biol. Cell* **23**, 2782–2792 (2012).
- B. J. Ballew *et al.*, *Hum. Genet.* **132**, 473–480 (2013).
- A. J. Walne, T. Vulliamy, M. Kirwan, V. Plagnol, I. Dokal, *Am. J. Hum. Genet.* **92**, 448–453 (2013).
- T. Le Guen *et al.*, *Hum. Mol. Genet.* **22**, 3239–3249 (2013).
- K. M. Egan *et al.*, *J. Neurooncol.* **104**, 535–542 (2011).
- S. Shete *et al.*, *Nat. Genet.* **41**, 899–904 (2009).
- T. Jacks *et al.*, *Curr. Biol.* **4**, 1–7 (1994).
- R. J. Gilbertson, D. W. Ellison, *Annu. Rev. Pathol.* **3**, 341–365 (2008).

Acknowledgments: We thank the Protein Analysis and Proteomics group at London Research Institute for mass spectrometry and J. Mendez for MCM2 antibody. Research in the DNA damage response lab of S.J.B. is funded by Cancer Research UK and by a European Research Council (ERC) Advanced Investigator Grant (RecMitMei). S.J.B. is a recipient of a Royal Society Wolfson Research Merit Award. The laboratory of H.D. is supported by Canada Research Chair program, Canada Institute of Health Research, Manitoba Institute of Child Health, and Terry Fox Research Institute. J.-B.V. is funded by a long-term fellowship from ERC, and S.S. and Z.N. are supported by the fellowships from Natural Sciences and Engineering Research Council of Canada (to S.S.) and the Manitoba Health Research Council (to Z.N.).

Supplementary Materials

www.sciencemag.org/content/342/6155/239/suppl/DC1
Materials and Methods
Figs. S1 to S7
Table S1
References (19–26)

11 June 2013; accepted 11 September 2013
10.1126/science.1241779

Structures and Receptor Binding of Hemagglutinins from Human-Infecting H7N9 Influenza Viruses

Yi Shi,^{1,2*} Wei Zhang,^{2,3*} Fei Wang,^{2,4*} Jianxun Qi,^{2*} Ying Wu,^{2*} Hao Song,^{2,3} Feng Gao,⁵ Yuhai Bi,² Yanfang Zhang,⁶ Zheng Fan,⁷ Chengfeng Qin,⁸ Honglei Sun,⁴ Jinhua Liu,⁴ Joel Haywood,² Wenjun Liu,² Weimin Gong,⁵ Dayan Wang,⁹ Yuelong Shu,⁹ Yu Wang,¹⁰ Jinghua Yan,² George F. Gao^{1,2,3,4,6,9,10†}

An avian-origin human-infecting influenza (H7N9) virus was recently identified in China. We have evaluated the viral hemagglutinin (HA) receptor-binding properties of two human H7N9 isolates, A/Shanghai/1/2013 (SH-H7N9) (containing the avian-signature residue Gln²²⁶) and A/Anhui/1/2013 (AH-H7N9) (containing the mammalian-signature residue Leu²²⁶). We found that SH-H7N9 HA preferentially binds the avian receptor analog, whereas AH-H7N9 HA binds both avian and human receptor analogs. Furthermore, an AH-H7N9 mutant HA (Leu²²⁶ → Gln) was found to exhibit dual receptor-binding property, indicating that other amino acid substitutions contribute to the receptor-binding switch. The structures of SH-H7N9 HA, AH-H7N9 HA, and its mutant in complex with either avian or human receptor analogs show how AH-H7N9 can bind human receptors while still retaining the avian receptor-binding property.

In February 2013, a novel reassortant influenza A (H7N9) virus was identified in eastern China, which by 30 April had spread to more than 11 provinces and municipalities. This virus is a low-pathogenicity avian influenza (LPAI) virus in domestic poultry (1–4). Until now, only sporadic cases of severe human infection with an LPAI virus have been reported (5, 6). Understanding the underlying mechanism of the avian-human host “jump” is crucial for the development of effective preventive and therapeutic measures (7–12).

The viral surface glycoprotein hemagglutinin (HA) is responsible for host receptor binding and is the major determinant of the virus host “jump” (13). Recent work has shown that the Gln²²⁶ → Leu (Q226L; H3 numbering used throughout) substitution in avian H5N1 HA confers human receptor (α -2,6-linked galactose) binding and simultaneously reduces avian receptor (α -2,3-linked galactose) binding (14–17). The observation of a receptor shift in influenza viruses creates concern

that a pandemic in human beings might begin this way (14–17). It is noteworthy that H7N9 HA has a naturally occurring Q226L substitution observed in most of the isolates [e.g., AH-H7N9, A/Anhui/1/2013 (H7N9)], with the exception of an earlier Shanghai isolate that retained the gluta-

mine at position 226 [SH-H7N9, A/Shanghai/1/2013 (H7N9)] (8). These findings have led to the assumption that the AH-H7N9 lineage virus might have acquired high-affinity human receptor-binding properties.

To characterize the receptor-binding properties of AH-H7N9 and SH-H7N9 at the virus level, we rescued the viruses with reverse genetics technology (18); the rescued viruses were named rAH-H7N9 and rSH-H7N9. We analyzed their receptor-binding properties through solid-phase binding assays using the 2009 pandemic influenza virus isolate [CA04-H1N1, A/California/04/2009 (H1N1)] and avian H5N1 influenza virus isolate [AH05-H5N1, A/Anhui/1/2005 (H5N1)] as control viruses that have typical human or avian receptor specificity, respectively. rAH-H7N9 binds both the human and avian receptor, whereas rSH-H7N9 preferentially binds the avian receptor (Fig. 1, A and B). In contrast, CA04-H1N1 specifically binds the human receptor (Fig. 1C), and AH05-H5N1 specifically binds the avian receptor (Fig. 1D).

To further evaluate the binding affinities of AH-H7N9 and SH-H7N9 to canonical avian-like and human-like receptor analogs (18), we prepared soluble HA proteins for both viruses and showed by surface plasmon resonance (SPR) experiments that, similar to the rescued viruses, AH-H7N9 HA binds both avian- and human-like receptors, whereas

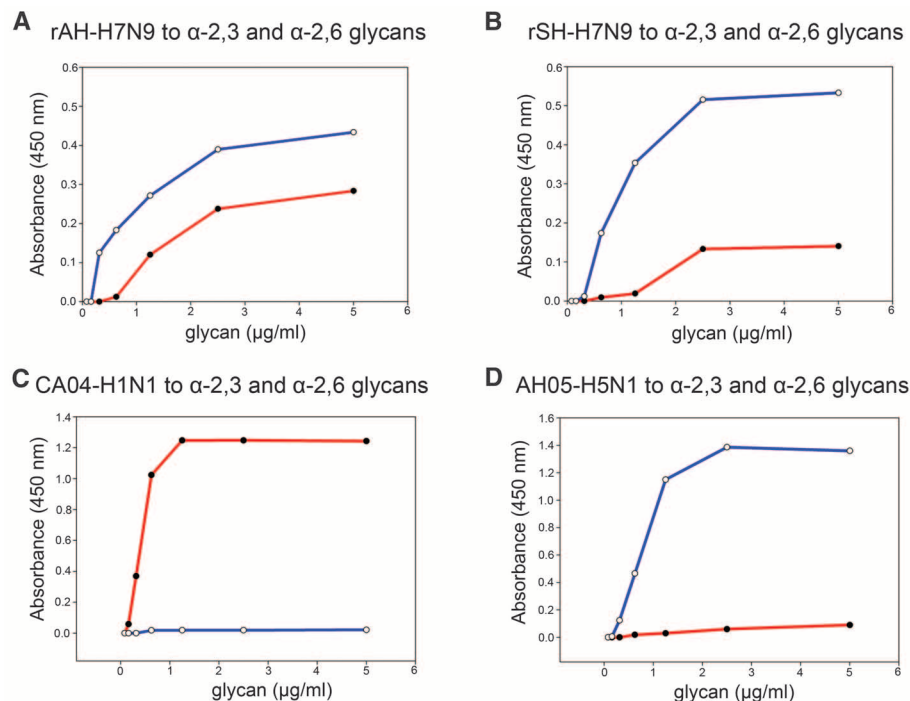


Fig. 1. Receptor-binding properties at virus level. Binding of virus to α -2,3-linked (3'SLNLN) or α -2,6-linked (6'SLNLN) sialylglycan receptors was determined by solid-phase binding assays. (A) rAH-H7N9 (reverse genetics–rescued A/Anhui/1/2013) virus; (B) rSH-H7N9 (reverse genetics–rescued A/Shanghai/1/2013) virus; (C) CA04-H1N1 (A/California/04/2009) virus; (D) AH05-H5N1 (A/Anhui/1/2005) virus. Blue, binding to 3'SLNLN; red, binding to 6'SLNLN. rAH-H7N9 binds to both 3'SLNLN and 6'SLNLN, whereas rSH-H7N9 binds preferentially to 3'SLNLN. As a control, CA04-H1N1 specifically binds 6'SLNLN, and AH05-H5N1 specifically binds 3'SLNLN.

¹Research Network of Immunity and Health, Beijing Institutes of Life Science, Chinese Academy of Sciences, Beijing 100101, China. ²CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China. ³University of Chinese Academy of Sciences, Beijing 100049, China. ⁴College of Veterinary Medicine, China Agricultural University, Beijing 100193, China. ⁵Laboratory of Non-coding RNA, Institute of Biophysics, Chinese Academy of Sciences, Datun Road, Beijing 100101, China. ⁶Laboratory of Protein Engineering and Vaccines, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China. ⁷Core Facility, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China. ⁸Department of Virology, Beijing Institute of Microbiology and Epidemiology, Beijing 100071, China. ⁹National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention (China CDC), Beijing 102206, China. ¹⁰Office of Director-General, China CDC, Beijing 102206, China.

*These authors contributed equally to this work.

†Corresponding author. E-mail: gaogf@mail.biols.ac.cn

SH-H7N9 HA binds only the avian-like receptor (Fig. 2, A to F). AH-H7N9 HA bound both avian and human receptors, with high affinities of 0.10 μ M and 0.33 μ M, respectively (Fig. 2, A to C). SH-H7N9 HA also showed a binding preference for the avian receptor (with a similarly high affinity of 0.16 μ M), but by contrast had extremely weak or no binding to the human receptor (>1 mM, beyond the SPR measurement range) (Fig. 2, D to F). The binding kinetics of SH-H7N9 HA are similar to the previously reported H7N7 HA from a highly pathogenic avian influenza (HPAI) A/H7N7 virus isolate (A/Netherlands/219/2003) (19). In contrast to the Q226L substitution of H5N1, which has endowed this virus with the ability to bind to human receptors but reduced its affinity for avian receptors (14–17), AH-H7N9 HA has maintained dual receptor-binding properties despite having the same substitution.

In total, there are eight amino acid substitutions between the SH-H7N9 and AH-H7N9 HAs, of which four residues—S138A (Ser¹³⁸ → Ala), G186V (Gly¹⁸⁶ → Val), T221P (Thr²²¹ → Pro), and Q226L—locate in the receptor-binding

site (RBS) (fig. S1). There are likely several distinct mechanisms by which a shift in host receptor-binding preference can take place in different HA subtypes (20, 21). This is illustrated by differences between avian H1 and avian H2 and H3 viruses, in which the H2 and H3 viruses require Q226L and G228S (Gly²²⁸ → Ser) substitutions, whereas the H1 virus HA requires E190D (Glu¹⁹⁰ → Asp) and G225D (Gly²²⁵ → Asp) substitutions in the RBS, retaining the residue Gln²²⁶ (21–26). Thus, to investigate whether the Q226L substitution is a key determinant for obtaining the human receptor binding in H7, we introduced one L226Q substitution into AH-H7N9 HA, which binds the avian receptor analog with an affinity of 0.2 μ M, similar to that of wild-type AH-H7N9 HA. Surprisingly, it retained its ability to bind to the human receptor analog, albeit with a reduced affinity of 1.2 μ M (Fig. 2, G to I). Therefore, the other three amino acid substitutions are sufficient for human receptor binding for H7, without the Q226L substitution.

Using x-ray crystallography, we solved the structures of SH-H7N9 HA and of AH-H7N9 HA

and its mutant, all in their free form or in complex with the two sialo-pentasaccharides 3'SLNLN and 6'SLNLN. These sialo-pentasaccharides are analogs of the avian and human receptors, respectively, and contain the three terminal saccharides sialic acid (Sia), galactoside (Gal), and *N*-acetylglucosamine (GlcNAc) (27). Given the resolution of the structures (2.6 Å, 2.6 Å, 2.8 Å, 2.5 Å, 3.0 Å, and 3.1 Å, respectively) (tables S1 to S3), there is unambiguous electron density for the ligands in the six complexes (fig. S2).

The RBS is at the membrane-distal end of each monomer. Conventionally, the RBS of H7 is divided into two parts: (i) the base, consisting of the conserved residues Tyr⁹⁸, Trp¹⁵³, His¹⁸³, and Tyr¹⁹⁵; and (ii) the side, consisting of the secondary elements 130-loop, 190-helix, and 220-loop. The structure of AH-H7N9 HA in complex with the avian receptor analog 3'SLNLN revealed that the analog bound in a cis conformation (Fig. 3A), similar to that seen in two recently reported H5 mutants in complex with the avian receptor analogs (16, 17, 28). However, there were clear differences of the molecular interactions with respect

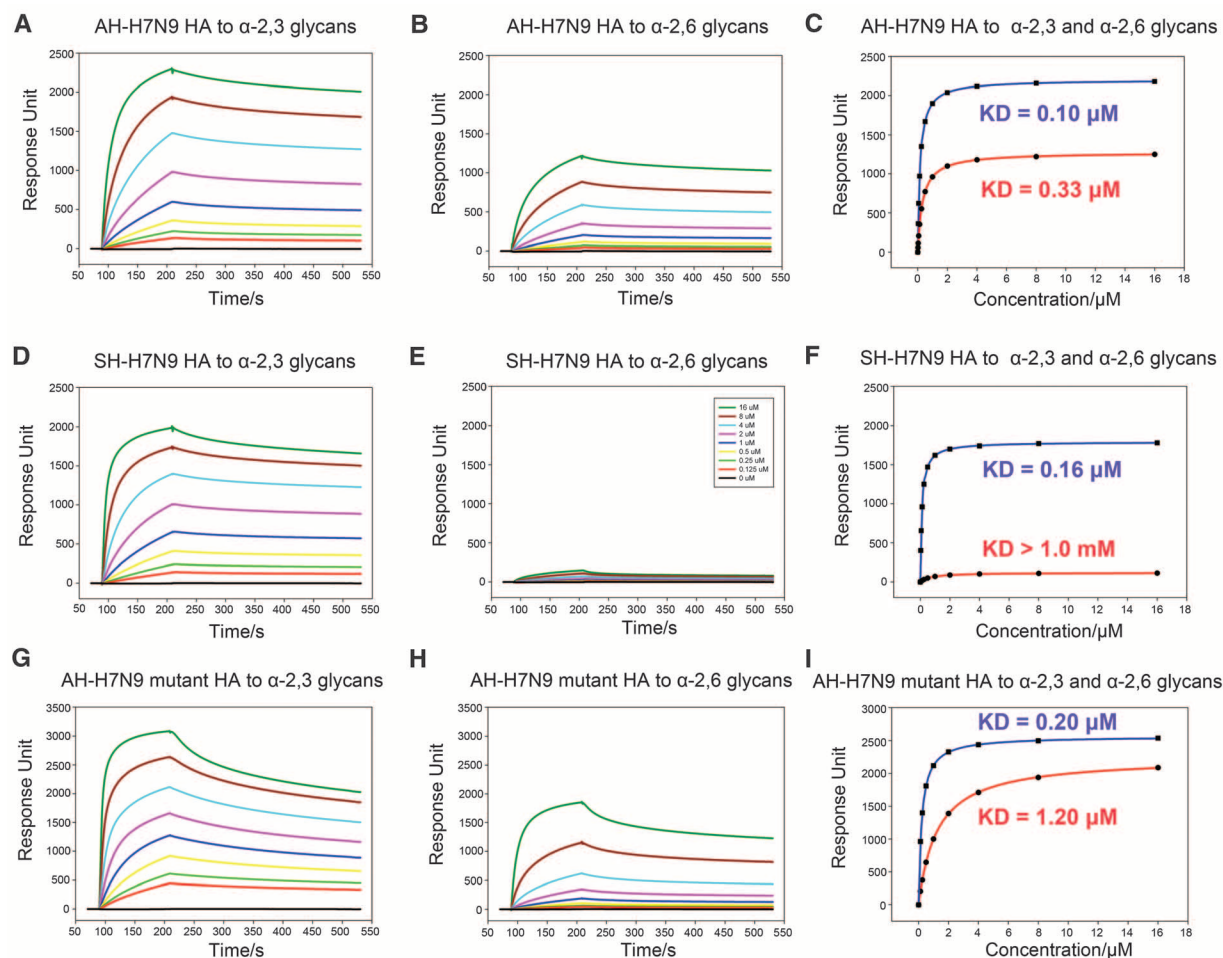


Fig. 2. Receptor-binding properties at protein level. (A, B, D, E, G, and H) BIAcore plots showing binding of AH-H7N9 HA to 3'SLNLN (A) and 6'SLNLN (B), binding of SH-H7N9 HA to 3'SLNLN (D) and 6'SLNLN (E), and binding of AH-H7N9 mutant HA to 3'SLNLN (G) and 6'SLNLN (H). (C, F, and I) Response

units were plotted against protein concentrations. Blue, binding to 3'SLNLN; red, binding to 6'SLNLN. The binding affinity (K_D) values were calculated using a steady-state affinity model produced with BIAcore 3000 analysis software (BIAevaluation Version 4.1).

to the H5 mutants. In addition to the seven conserved hydrogen bond interactions between Sia-1 and RBS residues, the adjacent glycan rings (Gal-2 and GlcNAc-3) formed substantive van der Waals interactions with the 220-loop in the AH-H7N9 HA–avian receptor complex (Fig. 3A), which has not been observed in other HA–avian receptor complexes. Crucially, the main-chain carbonyl oxygen of Gly²²⁵ formed one hydrogen bond with Gal-2, and the side chain of Gln²²⁶ formed a hydrogen bond with GlcNAc-3, which stabilized the conformations of both rings. Moreover, the residue Leu²²⁶ created a hydrophobic environment for the nonpolar portion of Gal-2. Interestingly, in the presence of Gln²²⁶, SH-H7N9 HA also bound avian receptor analog in a cis conformation, in a manner similar to that of avian receptor analog bound to AH-H7N9 HA (Fig. 3B). In contrast, although exhibiting the same Gln²²⁶, the AH-H7N9 mutant HA bound the avian receptor analog in a trans conformation (Fig. 3C), which is usually observed in other avian HA–avian receptor complexes. This indicates that the amino acid at position 226 is not a key determinant for the conformation of avian receptor

binding in the H7 subtype. In fact, both cis and trans configurations are in low-energy conformations for α -2,3 linkages, and both have been observed in studies of sialosides in solution and bound to other proteins (29–34). We have captured two conformations of avian receptor binding in H7 HAs with a Gln²²⁶ in this study.

The structure of AH-H7N9 HA in complex with the human receptor analog 6'SLNLN showed that the analog was bound in a cis conformation (Fig. 3D). The residue Leu²²⁶ formed van der Waals interactions with C α atoms of Gal-2 around the glycosidic linkage, and the main-chain carbonyl oxygen of residue Gly²²⁵ formed one hydrogen bond with the 3-OH of Gal-2 (Fig. 3D). In the SH-H7N9 HA–human receptor complex, only the sialic acid moiety was observed and the remaining four glycan rings of the pentasaccharide were not visible (Fig. 3E), implying a weak interaction as observed in the SPR experiment. The AH-H7N9 mutant HA bound the human receptor in a cis conformation, and the Gln²²⁶ formed two hydrogen bonds with sialic acid and one hydrogen bond with Gal-2 (Fig. 3F). Although the Leu²²⁶ was substituted as Gln²²⁶ in the AH-H7N9 mutant

HA, the other three residues (Ala¹³⁸, Val¹⁸⁶, and Pro²²¹) were able to create a hydrophobic region (fig. S3) in the RBS, allowing the human receptor to bind in a cis conformation.

Structural comparison revealed that a similar cis conformation occurred when SH-H7N9 HA and AH-H7N9 HA bound to an avian receptor analog, where the glycan moieties sat lower on the 220-loop (by ~ 2 Å) in SH-H7N9 HA than in AH-H7N9 HA (Fig. 4A). We assume that the four hydrophilic residues (Ser¹³⁸, Gly¹⁸⁶, Thr²²¹, and Gln²²⁶) of SH-H7N9 HA are more compatible for the hydrophilic glycosidic oxygen of the avian receptor analog than the four hydrophobic residues (Ala¹³⁸, Val¹⁸⁶, Pro²²¹, and Leu²²⁶) of AH-H7N9 HA (fig. S3). The trans conformation of avian receptor analog binding was observed in the AH-H7N9 mutant HA complex (Fig. 4B), and structural comparison revealed a $\sim 180^\circ$ rotation around the glycosidic linkage between the AH-H7N9 mutant HA complex and the AH-H7N9 or SH-H7N9 HA complex (Fig. 4, B and C). The residues Gly²¹⁶ and Gln²¹³ further stabilized the conformations of Gal-2 and GlcNAc-3, and dragged the two glycan rings toward the 220-loop by ~ 4 Å

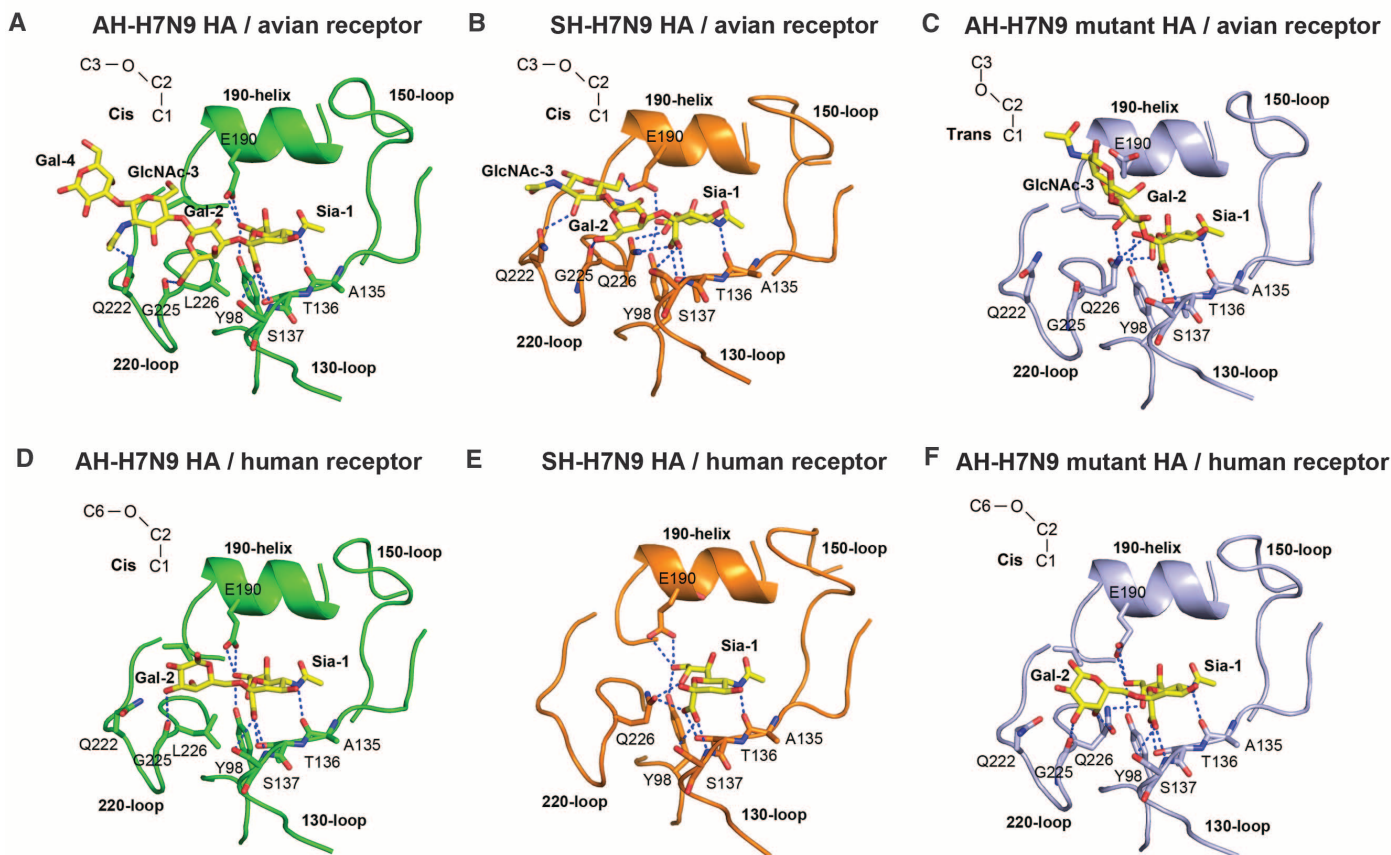


Fig. 3. Structural comparative analyses of the interactions of AH-H7N9 HA, SH-H7N9 HA, and AH-H7N9 mutant HA with either avian or human receptor analogs. The three secondary structural elements of the binding site (i.e., the 130-loop, 190-helix, and 220-loop; H3 numbering) are labeled in ribbon representation, together with the selected residues in stick representation. Hydrogen bonds are shown as dashed lines. Green, AH-H7N9 HA; orange, SH-H7N9 HA; light blue, AH-H7N9 mutant HA; yellow, glycans. (A to C) AH-H7N9 HA (A),

SH-H7N9 HA (B), and AH-H7N9 mutant HA (C) with the avian receptor analogs bound. The receptor analogs bind in different conformations in AH-H7N9 HA (cis), SH-H7N9 HA (cis), and AH-H7N9 mutant HA (trans). (D to F) AH-H7N9 HA (D), SH-H7N9 HA (E), and AH-H7N9 mutant HA (F) with the human receptor analogs bound. For AH-H7N9 HA, the receptor analog binds in a cis conformation. Only the sialic acid moiety is observed in the SH-H7N9 HA–human receptor complex. For AH-H7N9 mutant HA, the receptor analog binds in a cis conformation.

or ~ 6 Å, respectively (Fig. 3, A and C, and Fig. 4, B and C).

By contrast, the four hydrophilic residues (Ser¹³⁸, Gly¹⁸⁶, Thr²²¹, and Gln²²⁶) in SH-H7N9 HA are expected to create an unfavorable environment for the nonpolar portion of the human receptor analog around the glycosidic linkage, which results in no binding to the human receptor. However, we found that when AH-H7N9 HA bound to the human receptor analog, the hydrophobic residues (Ala¹³⁸, Val¹⁸⁶, Pro²²¹, and Leu²²⁶) stabilized the cis conformation of the receptor analog by creating a favorable platform on which the Gal-2 can sit to form a tighter interaction between the receptor and the ligand. In the AH-H7N9 mutant HA complex, the Leu²²⁶ was substituted by Gln²²⁶, but the receptor analog still bound in a cis conformation, indicating that the other three hydrophobic residues maintained the hydrophobic interaction even in the absence of hydropho-

bic Leu²²⁶. Analysis of the human receptor analog showed that there is a 70° rotation around the Gal-2 C6-C5 bond between the AH-H7N9 HA and AH-H7N9 mutant HA complexes (Fig. 4D). Depending on the orientation of the Gal-2, the remaining three glycan rings of the pentasaccharide may be oriented in different directions (Fig. 4D). For example, the glycan receptor in the AH-H7N9 HA complex would extend from the RBS, whereas the glycan receptor in AH-H7N9 mutant HA complex would extend toward the space between the 190-helix and the 220-loop.

Previous studies have shown that the RBSs of the human and swine influenza virus HAs are larger than those of the avian influenza virus HAs (35). We compared the HAs of AH-H7N9, SH-H7N9, and the 1957 Singapore human H2N2 (57H2N2) to see whether AH-H7N9 HA had acquired similar characteristics. The distances between the 130-loop and the 220-loop of the RBS

are larger (by ~ 1.5 Å) in the AH-H7N9 HA structure than in the SH-H7N9 HA structure (Fig. 4E). By contrast, the distances are comparable between the AH-H7N9 mutant HA and SH-H7N9 HA (Fig. 4E). It is noteworthy that one hydrogen bond was formed between the residues Gln²²⁶ and Ser¹³⁸ in SH-H7N9 HA and that the 220-loop and 130-loop were connected tightly (fig. S3). In the AH-H7N9 mutant HA, as a result of the S138A substitution, the residue Gln²²⁶ was displaced by ~ 2 Å (fig. S3). The different residue interactions might be responsible for the different binding properties of AH-H7N9 mutant HA and SH-H7N9 HA. The distances between the 130-loop and the other two sides of the RBS (190-helix and 220-loop) are comparable in the AH-H7N9 HA and 57H2N2 HA structures (Fig. 4F). The 150-loop moves closer toward the 190-helix of RBS by >6 Å in the AH-H7N9 HA structure relative to the 57H2N2 HA structure (Fig. 4F). Previous

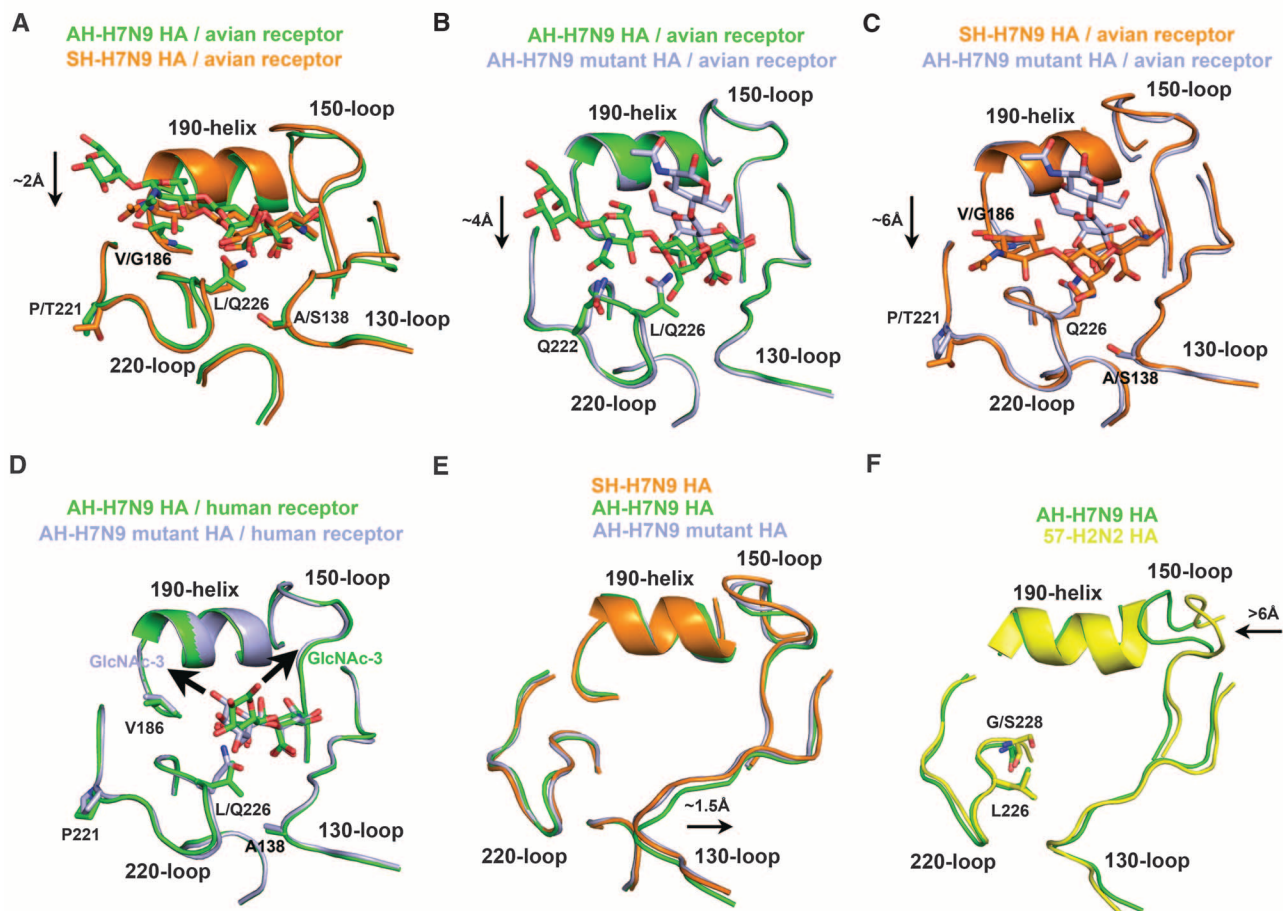


Fig. 4. Structural comparison of AH-H7N9 HA, SH-H7N9 HA, and AH-H7N9 mutant HA complexes. (A) Comparison of RBSs of the AH-H7N9 HA–avian receptor (green) and SH-H7N9 HA–avian receptor (orange) complexes. A similar cis conformation for glycan binding is observed between these two complexes, but the glycan receptor sits lower (by ~ 2 Å) in the SH-H7N9 complex. (B) Comparison of RBSs of the AH-H7N9 HA–avian receptor (green) and AH-H7N9 mutant HA–avian receptor (light blue) complexes. The glycan receptor sits lower (by ~ 4 Å) in the AH-H7N9 HA complex. (C) Comparison of RBSs of the SH-H7N9 HA–avian receptor (orange) and AH-H7N9 mutant HA–avian receptor (light blue) complexes. The glycan

receptor sits lower (by ~ 6 Å) in the SH-H7N9 HA complex. (D) Comparison of RBSs of the AH-H7N9 HA–human receptor (green) and AH-H7N9 mutant HA–human receptor (light blue) complexes. There is a 70° rotation around the Gal-2 C6-C5 bond. (E) Comparison of RBSs of AH-H7N9 HA (green), SH-H7N9 HA (orange), and AH-H7N9 mutant HA (light blue). The RBS of AH-H7N9 HA is wider than those of SH-H7N9 HA and AH-H7N9 mutant HA. (F) Comparison of RBSs of AH-H7N9 HA (green) and 57H2N2 HA (yellow). AH-H7N9 HA has a similarly wide RBS relative to 57H2N2 HA (PDB code 2WR7), but the 150-loop of AH-H7N9 HA is much closer to the RBS (by >6 Å) than that of 57H2N2 HA.

modeling suggests that longer α -2,6 linkage receptor analogs may clash with the 150-loop (36), and such loop interference probably causes multiple conformations of the glycan rings. This might explain why we obtained a poor electron density map for the glycan rings GlcNAc-3, Gal-4, and GlcNAc-5 of the human receptor analog 6'SLNLN in AH-H7N9 HA-human receptor complex.

Previous studies have shown that HA receptor binding of appropriate affinity and specificity is a requirement for efficient virus transmission between individuals and between species (16, 17, 37). The loss of affinity for the avian receptor appears to be an important factor for efficient human-to-human transmission; however, to date, limited human-to-human transmission has been observed for H7N9, which might be a result of retention of high affinity for the avian receptor. Maintenance of avian receptor binding can trap the virus in the human upper airways, which contain mucin molecules rich in α -2,3-linked galactose (38); this in turn leads to a requirement of high-dose virus to reach the susceptible cells, making human-to-human transmission more difficult. Anhui Leu²²⁶-containing virus binds human receptors with a greater affinity, and it dominates most of the later isolates. The earlier Shanghai Gln²²⁶-containing virus became less prominent, implying that the H7N9 virus is evolving. Furthermore, our mutagenesis study shows that, in contrast to H5N1 HA, the Q226L substitution is not solely responsible for the avian-to-human receptor-binding switch for H7 HA. Previous studies have also shown that many North American and Eurasian H7 influenza viruses display weak but detectable binding to the human-type receptor, highlighting the potential of H7 influenza viruses for avian-to-human transmission (39). We believe that surveillance of H7N9 virus isolates for detection of

the new amino acid substitutions is essential for the future implementation of control strategies.

References and Notes

1. J. C. de Jong, E. C. Claas, A. D. Osterhaus, R. G. Webster, W. L. Lim, *Nature* **389**, 554 (1997).
2. R. A. Fouchier *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **101**, 1356–1361 (2004).
3. M. Koopmans *et al.*, *Lancet* **363**, 587–593 (2004).
4. J. A. Belser *et al.*, *J. Virol.* **87**, 5746–5754 (2013).
5. M. Hirst *et al.*, *Emerg. Infect. Dis.* **10**, 2192–2195 (2004).
6. J. S. Nguyen-Van-Tam *et al.*, *Euro Surveill.* **11**, E0605042 (2006); www.eurosurveillance.org/ViewArticle.aspx?ArticleId=2952.
7. Y. Chen *et al.*, *Lancet* **381**, 1916–1925 (2013).
8. R. Gao *et al.*, *N. Engl. J. Med.* **368**, 1888–1897 (2013).
9. Q. Li *et al.*, *N. Engl. J. Med.* **368**, 1304617 (2013).
10. D. Liu *et al.*, *Lancet* **381**, 1926–1932 (2013).
11. Y. Wu, G. F. Gao, *Sci. China Life Sci.* **56**, 493–494 (2013).
12. J. Li *et al.*, *Sci. China Life Sci.* **56**, 485–492 (2013).
13. J. J. Skehel, D. C. Wiley, *Annu. Rev. Biochem.* **69**, 531–569 (2000).
14. S. Herfst *et al.*, *Science* **336**, 1534–1541 (2012).
15. M. Imai *et al.*, *Nature* **486**, 420–428 (2012).
16. X. Xiong *et al.*, *Nature* **497**, 392–396 (2013).
17. W. Zhang *et al.*, *Science* **340**, 1463–1467 (2013).
18. See supplementary materials on Science Online.
19. H. Yang, P. J. Carney, R. O. Donis, J. Stevens, *J. Virol.* **86**, 8645–8652 (2012).
20. W. Zhang *et al.*, *J. Virol.* **87**, 5949–5958 (2013).
21. S. J. Gamblin *et al.*, *Science* **303**, 1838–1842 (2004).
22. G. N. Rogers *et al.*, *Nature* **304**, 76–78 (1983).
23. G. N. Rogers *et al.*, *J. Biol. Chem.* **260**, 7362–7367 (1985).
24. A. S. Gambaryan *et al.*, *Virology* **232**, 345–350 (1997).
25. M. Matrosovich *et al.*, *J. Virol.* **74**, 8502–8512 (2000).
26. J. Stevens *et al.*, *J. Mol. Biol.* **355**, 1143–1155 (2006).
27. M. B. Eisen, S. Sabesan, J. J. Skehel, D. C. Wiley, *Virology* **232**, 19–31 (1997).
28. X. Lu *et al.*, *Protein Cell* **7**, 502–511 (2013).
29. J. Breg, L. M. Kroon-Batenburg, G. Strecker, J. Montreuil, J. F. Vliegthart, *Eur. J. Biochem.* **178**, 727–739 (1989).
30. L. Poppe, R. Stuike-Prill, B. Meyer, H. van Halbeek, *J. Biomol. NMR* **2**, 109–136 (1992).

31. S. Sabesan, K. Bock, J. C. Paulson, *Carbohydr. Res.* **218**, 27–54 (1991).
32. T. Stehle, S. C. Harrison, *Structure* **4**, 183–194 (1996).
33. C. S. Wright, J. Jaeger, *J. Mol. Biol.* **232**, 620–638 (1993).
34. E. A. Merritt *et al.*, *Protein Sci.* **3**, 166–175 (1994).
35. Y. Ha, D. J. Stevens, J. J. Skehel, D. C. Wiley, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 11181–11186 (2001).
36. R. J. Russell, D. J. Stevens, L. F. Haire, S. J. Gamblin, J. J. Skehel, *Glycoconj. J.* **23**, 85–92 (2006).
37. K. Srinivasan, R. Raman, A. Jayaraman, K. Viswanathan, R. Sasisekharan, *PLOS ONE* **8**, e49597 (2013).
38. J. N. Couceiro, J. C. Paulson, L. G. Baum, *Virus Res.* **29**, 155–165 (1993).
39. A. S. Gambaryan *et al.*, *J. Virol.* **86**, 4370–4379 (2012).

Acknowledgments: Supported by the China Ministry of Science and Technology National 973 Project (grant 2011CB504703), the National Natural Science Foundation of China (NSFC, grant 81290342), and Chinese Academy of Sciences intramural special grant KSZD-EW-Z-002 for influenza virus research. G.F.G. is a leading principal investigator of the NSFC Innovative Research Group (grant 81021003). We thank E. Dong for NSFC Medical Science Department director's special grant 81341002; staff at the Shanghai Synchrotron Radiation Facility (beamline 17U), especially J. He and S. Huang, for assistance; the Genewiz Corporation for rapid synthesis of HA genes; and the Consortium for Functional Glycomics for providing the biotinylated SA analogs. Coordinates and structure factors are deposited in the Protein Data Bank (PDB) with the following codes: 4KOL (AH-H7N9 HA), 4KOM (AH-H7N9 HA-3'SLNLN), 4KON (AH-H7N9 HA-6'SLNLN), 4LCX (SH-H7N9 HA), 4LKG (SH-H7N9 HA-3'SLNLN), 4LKH (SH-H7N9 HA-6'SLNLN), 4LKI (AH-H7N9 mutant HA), 4LKJ (AH-H7N9 mutant HA-3'SLNLN), and 4LKK (AH-H7N9 mutant HA-6'SLNLN).

Supplementary Materials

www.sciencemag.org/content/342/6155/243/suppl/DC1
Materials and Methods
Figs. S1 to S3
Tables S1 to S3
References (40–50)

6 May 2013; accepted 27 August 2013
Published online 5 September 2013;
10.1126/science.1242917

Human Influences on Nitrogen Removal in Lakes

Jacques C. Finlay,* Gaston E. Small,† Robert W. Sterner

Human activities have increased the availability of reactive nitrogen in many ecosystems, leading to negative impacts on human health, biodiversity, and water quality. Freshwater ecosystems, including lakes, streams, and wetlands, are a large global sink for reactive nitrogen, but factors that determine the efficacy of freshwater nitrogen removal rates are poorly known. Using a global lake data set, we show that the availability of phosphorus, a limiting nutrient, affects both annual nitrogen removal rate and efficiency. This result indicates that increased phosphorus inputs from human activities have stimulated nitrogen removal processes in many lakes. Recent management-driven reductions in phosphorus availability promote water column accumulation and export of nitrogen from large lakes, an unintended consequence of single-element management that argues for greater control of nitrogen as well as phosphorus sources.

Excess reactive nitrogen (N) from fertilizer use, crop N fixation, fossil fuel combustion, and other sources has led to large changes in ecosystems, including shifts in species composition, reduced biodiversity, and air and water quality impairment (1–3). Processes such as de-

nitrification and long-term storage (burial) in aquatic ecosystems remove substantial fractions of the total N inputs to watersheds (4) and thus provide a valuable ecosystem service by mitigating the impacts of increased human N inputs. A large but variable proportion of aquatic N re-

moval occurs in freshwater ecosystems, including groundwater, wetlands, streams, and lakes (5). Greater understanding of freshwater N removal is required to make effective management decisions to maintain and enhance N removal processes in the face of agricultural intensification and expansion, urbanization, and overall population growth that are increasing water quality degradation (5).

We synthesized annual ecosystem-scale mass balance measurements to explore the factors that are associated with permanent N removal in lakes, a globally important yet highly variable sink for N in landscapes (6). To interpret variation in permanent aquatic N removal (defined here as denitrification plus long-term sedimentary burial of organic N), we analyzed physical and biological factors known to affect N cycling and transport, focusing on factors that co-occur with increased

Department of Ecology, Evolution and Behavior, University of Minnesota, St. Paul, MN 55108, USA.

*Corresponding author. E-mail: jfinlay@umn.edu

†Present address: Department of Biology, University of St. Thomas, St. Paul, MN 55105, USA.

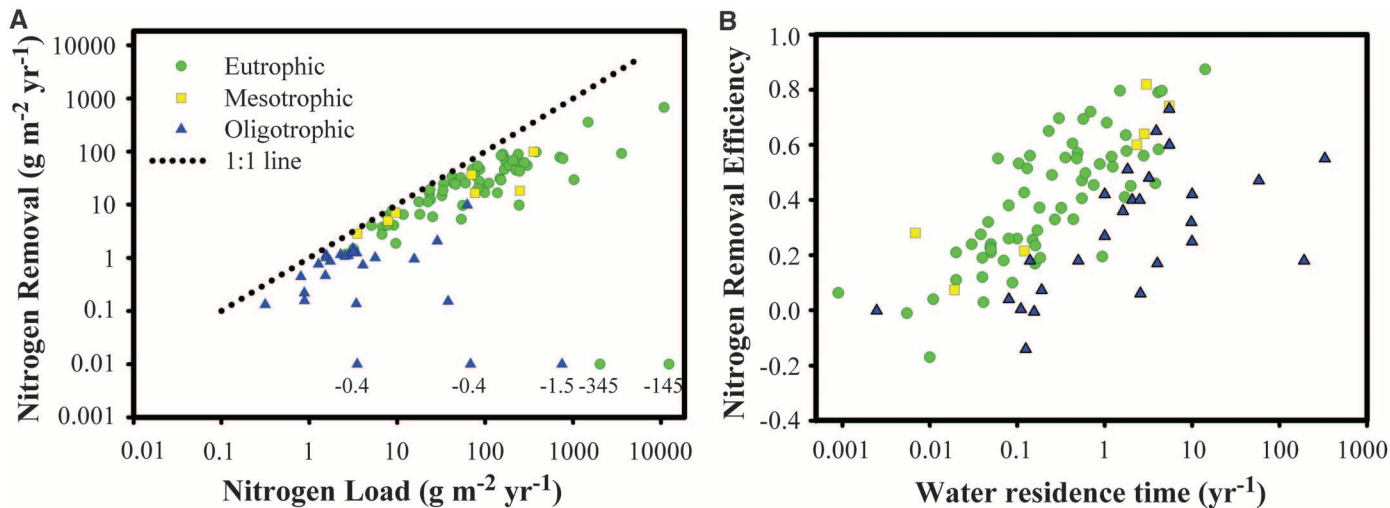


Fig. 1. Major influences on N removal and removal efficiency in lakes and reservoirs. (A) Annual rates of permanent TN removal related to annual N load and site trophic status. (B) Efficiency of TN removal as a function of water residence time and trophic status. Values of 0.01 in (A) were negative,

were assumed to be near zero for oligotrophic lakes, and were excluded for the two eutrophic lakes (supplementary text). The actual values are indicated in parentheses. Regression relationships for each lake type are presented in table S2.

Table 1. Models for N retention in lakes selected using Akaike’s information criterion (AIC_c) with correction for finite sample sizes [AIC_c (27)]. Effects of N loading (N load), water residence time (R), and TP and their interactive influences were examined with AIC_c for relationships to total N removal. For NRE, load was not included in the model based on AIC_c. *r*² adj., *r*² adjusted for the number of terms included in the model.

Model	<i>r</i> ² adj.	Factors	AIC _c
Specific TN retention (<i>n</i> = 85 sites)			
N load, TP, R, N load × TP, Load × R	0.82	6	72.7
N load, TP, R, N load × TP	0.79	5	83.7
N load, TP, R	0.78	4	88.6
N load, TP	0.75	3	95.5
TP	0.62	2	132.2
Intercept	0.0	1	212.3
Ecosystem TN retention efficiency (<i>n</i> = 85 sites)			
R, TP, R × TP	0.54	4	−69.5
R, TP	0.48	3	−59.8
R	0.30	2	−35
Intercept	0.00	1	−7.7

external N loading to lakes (supplementary text). In particular, we examined the role of phosphorus (P), a key limiting element in lakes that also has been strongly influenced by human activities (7) and is an important factor in widespread eutrophication in fresh water (8). Across a diverse and broadly representative set of lakes (supplementary text and table S3), total N (TN) removal rates (grams of N m^{−2} year^{−1}) increased with total external TN loading (sum of direct deposition, streams and rivers, and groundwater) (log TN_{removal} = −0.27 + 0.82 × log TN_{load}; *r*² = 0.78) and maintained high removal even under the highest TN loading rates (Fig. 1A). Ecosystem N removal efficiency (NRE, the proportion of TN inputs removed via denitrification or per-

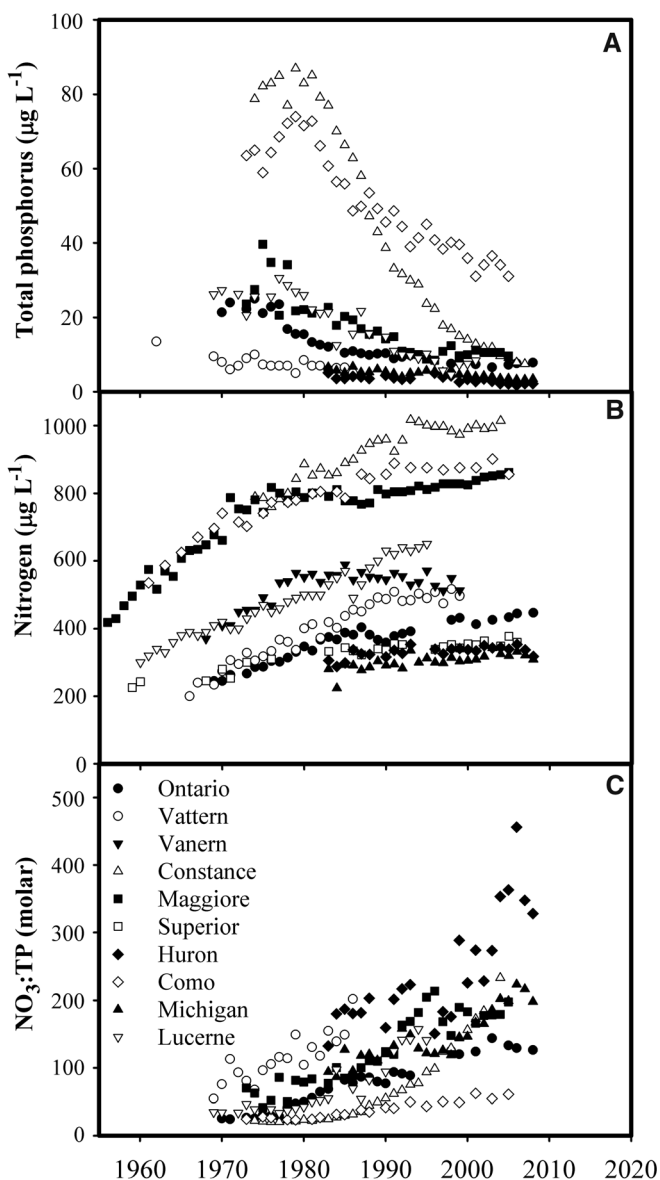
manent burial) was primarily influenced by water residence time, with longer residence times resulting in increased NRE, and was affected little by N loading rate (Table 1, table S1, and Fig. 1B). These analyses show that lakes can remove a large proportion of TN inputs (up to 90%) from surrounding watersheds, providing important water quality benefits to downstream ecosystems. Although TN loading and water residence time had large influences on TN removal rates and NRE, respectively, as observed previously (9, 10), the availability of P also was found to be an important determinant of N fate (Fig. 1, Table 1, and tables S1 and S2). For similar TN load and water residence time, P-rich eutrophic lakes removed over seven times more N than

oligotrophic lakes and were almost three times more efficient in removal of TN loads than were unproductive lakes (Fig. 1). The observed influence of P on nitrogen removal is mediated via coupled processes regulating the transfer of N from the water column to anoxic lake sediments (11) that promote N losses via denitrification (9). Decades of previous research have documented the relationships between P and individual factors that influence N cycling. Briefly, increased P stimulates algal production and inorganic N uptake (8), which in turn increase the transfer of N and particulate organic matter to lake sediments (12). This, in turn, drives increased heterotrophic metabolism and N mineralization in sediments and decreased dissolved oxygen concentrations, which increase denitrification rates (5). The effects of increased P on N removal are therefore mediated through interaction with at least two other elements (oxygen and carbon) and are the result of both water column and sedimentary processes in lakes. Additions of P have been demonstrated to increase N removal in whole-ecosystem experiments in both lakes and streams (13–15), providing further support for the role of P as an important control over N cycling and fate in freshwater ecosystems. Consideration of stoichiometric relationships among reactive elements will enhance our power to predict the fate of N in a wide range of complex ecosystems, including estuaries and coastal zones, terrestrial ecosystems (16, 17), and lakes. The observed influence of P on ecosystem TN removal suggests that lake water column N inventories should be responsive to changes in P availability and trophic status over time. To test this prediction, we examined long-term records of N and P concentration in 12 large lakes with high-resolution data sets and moderate to long water residence times (1 to 191 years; Table 2,

Table 2. Regression analyses of large lake N ($\mu\text{g of N year}^{-1}$) and P trends ($\log \mu\text{g of P year}^{-1}$). *ns*, not significant ($P > 0.05$). TP trends for Lakes Superior and Vanern were not included because of a lack of reliable P trend data (supplementary text).

Lake	Mean depth (m)	Water residence time (year^{-1})	N trend slope	Log P trend slope
Garda	133	26.6	<i>ns</i>	+0.019
Iseo	123	4.1	<i>ns</i>	+0.012
Huron	59	22	1.31	-0.012
Michigan	85	99	2.24	-0.010
Superior	147	191	2.78	*
Vanern	27	9.8	4.17	*
Ontario	85	6	5.04	-0.016
Maggiore	178	4.1	5.91	-0.018
Como	154	4.5	7.07	-0.014
Constance	100	4.3	8.26	-0.034
Vattern	40	58	9.12	-0.012
Lucerne	104	1	9.95	-0.019

Fig. 2. Management influences nutrient concentration in large lakes. Trends in water column (A) TP, (B) nitrate or dissolved inorganic N (DIN), and (C) $\text{NO}_3\text{:TP}$ or DIN:TP for large lakes with declining TP. TP and nutrient ratio data are not shown for Lake Superior, where TP is near the analytical detection limit, and Lake Vanern, where time series at two locations show poor correspondence and a weak negative trend. Decreased productivity related to declining P availability has been documented in most of these lakes, including Superior and Vanern (see supplementary text for details).



supplementary text). Many large lakes in developed watersheds have undergone steep declines in P availability and productivity in response to sewage input controls and watershed management, and N loading has also generally declined or stabilized (18, 19). However, despite reduced or stabilized N loading, N concentrations rose markedly (Fig. 2B) in 10 lakes that also exhibited decreasing total P (TP) or productivity (Fig. 2A and Table 2). These changes increased N:P ratios, indicating growing imbalances between P versus N availability for lake food webs (Fig. 2C). In contrast, large lakes without rising NO_3 trends did not show declining TP (Table 2). Together, these results show that large lakes accumulate N under conditions of imbalanced N:P availability.

The observed buildup of N in large lakes with decreasing P and productivity suggests that such systems increasingly lack the capacity to remove N inputs because of a weakened ability to assimilate N and transfer it to benthic denitrification zones. Important differences in the management and cycling of N versus P help explain the increasing mismatch between N and P availability. Controls of P availability in large lakes have been highly effective because of management focused on the reduction of watershed erosion and sewage, two primary human sources of P to aquatic ecosystems (7). In contrast, N management has been less effective because of diverse sources of nonpoint N inputs, including nitrate leaching from soils and atmospheric deposition, which are more difficult to control (20). In contrast to large lakes, smaller lakes show decreasing or stable NO_3 and TN trends during recent decades (18, 21). These divergent responses are probably due to rapid hydrologic turnover in small lakes, which obscures trends, as well as the higher availability of P and organic carbon (22) from surrounding watersheds, which may maintain high denitrification rates.

Our results suggest that the P enrichment often accompanying increased anthropogenic N loading has enhanced the metabolism and removal of N in lakes. Where increased N loads are stoichiometrically balanced with adequate P availability, the resulting human-driven enhancement of N removal may strongly reduce in-lake N concentrations and downstream export. The addition of excess N to unproductive P- and organic carbon-poor lakes leads to a stoichiometric imbalance that increases P limitation of phytoplankton and their consumers (23). Under these conditions, water column N accumulation and downstream flushing result, because these lakes lack an efficient mechanism to remove N. Data from large lakes as well as smaller, pristine, oligotrophic lakes strongly affected by atmospheric deposition support these predictions. Lakes with naturally low or reduced P availability (due to effective P trapping in sediments or management actions that have effectively reduced P) exhibit higher concentrations of nitrate than do more productive ones (24).

Excess P has many adverse effects on lakes, and its positive influence on N removal should therefore in no way be considered as a rationale for relaxing P control measures. Our results instead argue for increased attention to control of N sources from agricultural, urban, and fossil fuel sources, along with further reductions in P sources, to counteract negative impacts of N loading. The biological effects of rising N and N:P imbalances in lakes are incompletely known but may include reduced diversity and consumer growth rates (23). Furthermore, nitrate accumulation can eventually harm drinking water for human population centers that rely on lakes for water. Finally, accumulating N in lakes leads to increased fluvial export of N to downstream N-limited freshwater ecosystems and coastal zones, where it has been shown to stimulate algal blooms and drive a host of other water- and air-quality impairments (16, 25). Greater reduction of N loads via management changes will be difficult given that the sources of N are more diffuse than those of P, yet essential given the negative impacts of high aquatic N concentrations, the increasingly widespread implementation of P controls, and the

likelihood of sustained release of reactive N from agricultural intensification and fossil fuel combustion (2, 26).

References and Notes

- P. M. Vitousek *et al.*, *Ecol. Appl.* **7**, 737–750 (1997).
- J. N. Galloway *et al.*, *Science* **320**, 889–892 (2008).
- E. C. Suddick, P. Whitney, A. R. Townsend, E. A. Davidson, *Biogeochemistry* **114**, 1–10 (2013).
- W. H. Schlesinger, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 203–208 (2009).
- S. Seitzinger *et al.*, *Ecol. Appl.* **16**, 2064–2090 (2006).
- J. A. Harrison *et al.*, *Biogeochemistry* **93**, 143–157 (2009).
- D. W. Schindler, *Limnol. Oceanogr.* **51**, 356–363 (2006).
- D. W. Schindler, *Proc. Biol. Sci.* **279**, 4322–4333 (2012).
- D. L. Saunders, J. Kalff, *Hydrobiologia* **443**, 205–212 (2001).
- S. P. Seitzinger, *Limnol. Oceanogr.* **33**, 702–724 (1988).
- G. Small, J. Cotner, J. Finlay, R. Stark, R. Sterner, *Hydrobiologia* **10.1007/s10750-013-1569-7** (2013).
- G. Tartari, G. Biasci, *Water Air Soil Pollut.* **99**, 523 (1997).
- O. Kaste, A. Lyche-Solheim, *Can. J. Fish. Aquat. Sci.* **62**, 312–321 (2005).
- B. J. Peterson *et al.*, *Science* **229**, 1383–1386 (1985).
- W. Davidson, D. G. George, N. J. A. Edwards, *Nature* **377**, 504–507 (1995).
- R. Howarth *et al.*, *Front. Ecol. Environ.* **9**, 18–26 (2011).
- P. G. Taylor, A. R. Townsend, *Nature* **464**, 1178–1181 (2010).
- E. Jeppesen *et al.*, *Freshw. Biol.* **50**, 1747–1771 (2005).
- D. Gerdeaux, O. Anneville, D. Hefti, *Acta Oecol.* **30**, 161–167 (2006).
- S. R. Carpenter *et al.*, *Ecol. Appl.* **8**, 559–568 (1998).
- G. A. Weyhenmeyer *et al.*, *Limnol. Oceanogr.* **52**, 1346–1353 (2007).
- J. J. Cole *et al.*, *Ecosystems* **10**, 171–184 (2007).
- J. J. Elser *et al.*, *Science* **326**, 835–837 (2009).
- P. Hohener, R. Gächter, *Aquat. Sci.* **55**, 112–131 (1993).
- J. S. Baron *et al.*, *Biogeochemistry* **114**, 71–92 (2013).
- P. M. Vitousek *et al.*, *Science* **324**, 1519–1520 (2009).
- K. P. Burnham, D. R. Anderson, *Model Selection and Multi-Model Inference* (Springer, New York, ed. 2, 2002).

Acknowledgments: Data used in the analyses on nitrogen removal and the sources of these data are described in the supplementary materials (table S3). This research was supported by NSF under grant OCE-0927512.

Supplementary Materials

www.sciencemag.org/content/342/6155/247/suppl/DC1
Materials and Methods
Tables S1 to S3
References (28–86)

28 June 2013; accepted 4 September 2013
10.1126/science.1242575

Type 6 Secretion System–Mediated Immunity to Type 4 Secretion System–Mediated Gene Transfer

Brian T. Ho, Marek Basler, John J. Mekalanos*

Gram-negative bacteria use the type VI secretion system (T6SS) to translocate toxic effector proteins into adjacent cells. The *Pseudomonas aeruginosa* H1-locus T6SS assembles in response to exogenous T6SS attack by other bacteria. We found that this lethal T6SS counterattack also occurs in response to the mating pair formation (Mpf) system encoded by broad-host-range IncPα conjugative plasmid RP4 present in adjacent donor cells. This T6SS response was eliminated by disruption of Mpf structural genes but not components required only for DNA transfer. Because T6SS activity was also strongly induced by membrane-disrupting natural product polymyxin B, we conclude that RP4 induces “donor-directed T6SS attacks” at sites corresponding to Mpf-mediated membrane perturbations in recipient *P. aeruginosa* cells to potentially block acquisition of parasitic foreign DNA.

Bacteria often exhibit antagonistic behaviors toward each other in microbial communities (1). One molecular mechanism mediating such behavior is the type VI secretion system (T6SS) (2). The T6SS is a widely conserved (3) dynamic multicomponent nanomachine structurally related to contractile phage tails (4, 5). Gram-negative bacteria use the T6SS to kill prokaryotic and eukaryotic prey cells through contact-dependent delivery of toxic effectors (6, 7). In *P. aeruginosa*, T6SS encoded by the H1-T6SS

cluster (8) selectively targets T6SS⁺ bacteria that attack it by sensing these exogenous attacks and posttranslationally activating its own T6SS at the precise location of the initial assaults (9, 10). We previously hypothesized that the signal triggering the T6SS counterattack was the perturbation of the cell envelope (10). Thus, we wondered whether other systems capable of breaching the cell envelope would trigger a similar T6SS response. One system capable of delivering macromolecules across the envelopes of other Gram-negative cells is the type IV secretion system (T4SS) (11). This secretion system is associated with conjugative elements such as the broad-host-range, IncPα plasmid RP4 (12) as well as virulence elements in several bacterial species (13). T4SS-mediated DNA conjugation involves three sets of

proteins: (i) the core structure and pilus components comprising the mating pair formation (Mpf) complex, (ii) the relaxosome complex, which initiates DNA transfer by binding to and nicking the origin of transfer, and (iii) a coupling protein that bridges the relaxosome and Mpf complexes (14). During conjugation, the pilus extends from donor cells to mediate close cell-cell contact with recipients, which allows transfer of the DNA-bound relaxosome components to occur (14).

If T4SS-mediated cell-cell interactions could trigger T6SS attack, donor cells of a heterologous conjugation-proficient T6SS⁺ species should be sensitive to killing by T6SS⁺ *P. aeruginosa*. Therefore, we determined whether carrying the RP4 plasmid affected survival of *E. coli* K12 strain MC1061 when grown in competition with *P. aeruginosa*. For consistency with previous studies (9, 10), a *retS* mutant with a transcriptionally up-regulated H1-T6SS locus was used. When mixed with *P. aeruginosa*, we recovered ~96% fewer viable *E. coli* cells carrying RP4 as compared with those lacking it (Fig. 1A). This difference was not observed for *P. aeruginosa* mutants that were T6SS[−] (*vipA*) but was still observed in a triple mutant lacking the three known *P. aeruginosa* T6SS effectors Tse1, Tse2, and Tse3 (Fig. 1A) (7). Although a *pppA* mutant with a hyperactive but unregulated T6SS could slightly inhibit *E. coli* growth, there was no enhanced killing of *E. coli* cells carrying RP4 compared with those without it (Fig. 1A), and deletion of *tagT*, a gene critical for sensing exogenous T6SS attack (10), completely abolished *E. coli* killing (Fig. 1A). Furthermore, in a three-strain mixture containing RP4⁺ and RP4[−] *E. coli* with *P. aeruginosa*, only RP4⁺ *E. coli* were killed (Fig. 1B). Thus, T6SS-dependent killing of RP4⁺ *E. coli* involves

Department of Microbiology and Immunobiology, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA.

*Corresponding author. E-mail: john_mekalanos@hms.harvard.edu

the same attack-sensing mechanism implicated in the T6SS counterattack responses (10).

We next determined the genetic requirements for the RP4-dependent induction of the *P. aeruginosa* T6SS donor-directed attack. RP4 was subjected to transposon mutagenesis and transformed into *E. coli* strain MC1061. Individual mutants were sequenced to determine transposon insertion sites (Fig. 1C). Conjugation efficiency into recipient *E. coli* strain MG1655 was then determined for each of these RP4 mutants, and

T6SS activation efficiency was calculated from the survival rate of MC1061 *E. coli* with these mutant plasmids grown in competition with T6SS⁺ *P. aeruginosa* (table S1). Plotting the data for each mutant revealed several different phenotype clusters (Fig. 1D). Mutants in cluster 1 maintained wild-type levels of conjugation efficiency and induced T6SS killing at levels comparable with the wild-type plasmid. Most of these mutants were insertions in genes outside of the *traI* or *tra2* loci, the exceptions being a disruption in

the RP4 entry exclusion factor *trbK* (15) and a disruption of *traE*, a topoisomerase III homolog (16). Neither of these genes are required for the Mpf system or DNA transfer (table S1) (17). Mutants in cluster 2 were completely defective in their ability to transfer DNA and did not induce the T6SS donor-directed killing response in *P. aeruginosa*. All of these insertions disrupted genes encoding Mpf structural components (table S1). There were two outliers not quite in cluster 1 or 2 that were still able to transfer DNA but did

Fig. 1. Mpf induces a donor-directed T6SS attack in *P. aeruginosa*. (A) Summary of competition assays between either MC1061 (gray) or MC1061 RP4 (black) and the indicated strains of *P. aeruginosa*. Reported are the numbers of colony-forming units (CFUs) of surviving *E. coli*. Data are mean $\pm$ SD with $n = 4$ to 8 independent replicates. (B) Summary of 3-strain competitions between MC1061, MC1061 RP4 $\Delta traG$ (*Tra*[−], but *Mpf*⁺), and *P. aeruginosa*. Surviving CFUs of each *E. coli* strain determined by plating on media selective for each strain ($n = 6$ independent replicates). (C) Map of the RP4 plasmid indicating positions of transposon insertions. Labels with two genes separated by a slash (for example, *traE/traF*) represent insertions into the intergenic region between the two genes. (D) Plot of T6SS activation efficiency versus conjugation efficiency for each transposon mutant. Details on the indicated clusters are available in the text and table S1. Efficiencies are scaled so that values for wild-type RP4 are 100% and the RP4[−] parent strain are 0%. The blue dot represents wild-type RP4. The lower limit of detection for our assay was ~ 200 conjugants; mutants for which the conjugation efficiency was below this number are reported as 0% in the graph.

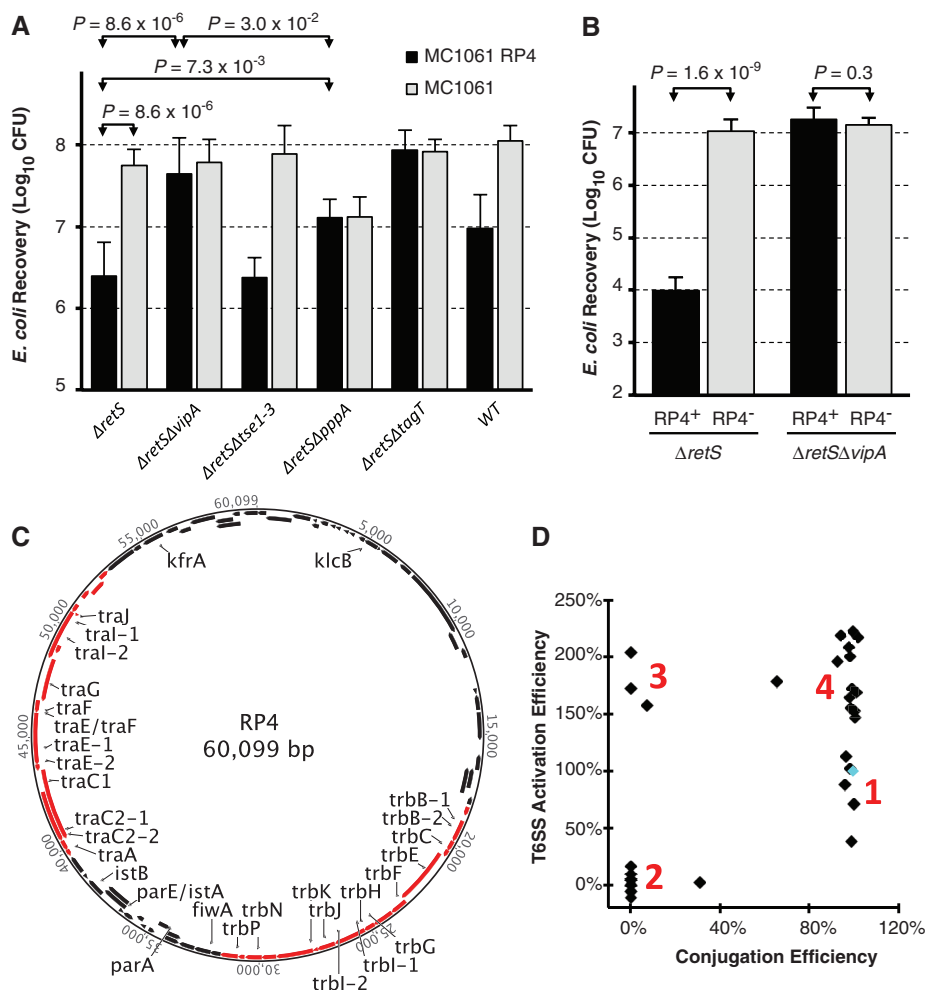
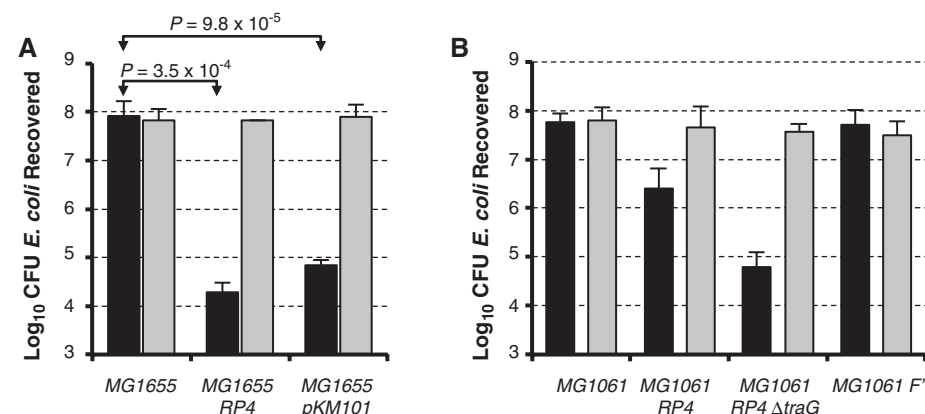


Fig. 2. IncN but not IncF induces donor-directed T6SS attacks. (A and B) Summary of *E. coli* survival after competition with T6SS⁺ (black bars) or T6SS[−] (gray bars) *P. aeruginosa*. Data are mean $\pm$ SD, $n = 3$ independent replicates. (A) Competition assays between *P. aeruginosa* and *E. coli* MG1655 carrying no plasmid, RP4, or pKM101. pKM101 confers streptomycin resistance, so MG1655 rather than MC1061 was used. (B) Competition assays between *P. aeruginosa* PAO1 and *E. coli* MC1061 carrying no plasmid, RP4, RP4 hyper-inducer *Tra*[−] *Mpf*⁺ mutant ($\Delta traG$), or F'. F' was confirmed to be functional by successfully mating into several different *E. coli* strains.



not induce a T6SS response (table S1): insertions in *trbH*, a lipoprotein believed to connect the pilus to the core complex (14); and *trbN*, a periplasmic transglycosylase that remodels the donor peptidoglycan and is required for pilus synthesis (14). Similar transposon disruptions of homologs of *trbH* (18) and *trbN* (19) in heterologous T4SSs affect the formation and stability of the Mpf pili. Mutants in cluster 3 induced a greater donor-directed T6SS response than that of wild-type RP4 but were defective in DNA conjugation (Fig. 2B). These mutants included disruptions of relaxo-

some components *traI* and *traJ* as well as coupling protein *traG* (table S1). Like those in cluster 3, mutants in cluster 4 also induced more T6SS killing than did the wild type but exhibited no defect in conjugation. Although it remains unclear why cluster-3 and -4 mutants induce more efficient T6SS-mediated killing, it is clear that successful DNA transfer is not required to trigger a T6SS attack by *P. aeruginosa*. We next determined whether other conjugative plasmids were also able to induce donor-directed T6SS attack. IncN compatibility group plasmid pKM101 (20) induced

a T6SS attack comparable with that of RP4 (Fig. 2A), whereas *E. coli* carrying the sex factor F plasmid was unaffected by T6SS⁺ *P. aeruginosa* (Fig. 2B). It is not known why the *E. coli* F factor cannot be successfully transferred into *P. aeruginosa* (21), but this observation suggests that T6SS activation correlates to some degree with whether the host range of a given plasmid includes *P. aeruginosa*.

If the *P. aeruginosa* donor-directed T6SS attack could be triggered by the Mpf system of donor species, then this attack might suppress plasmid transfer into a population of T6SS⁺ *P. aeruginosa* cells. Accordingly, we measured the frequency with which the plasmid pPSV35 (22) could be transferred into T6SS⁺ or T6SS⁻ *P. aeruginosa* from the *E. coli* donor strain SM10 (23), which carries a chromosomally integrated RP4 plasmid. Because pPSV35 does not encode its own transfer machinery but can be mobilized by the SM10-encoded conjugation system (22), the frequency with which *P. aeruginosa* cells acquired pPSV35 reflects the efficiency at which this plasmid is transferred into but not between *P. aeruginosa* cells. When donor *E. coli* and recipient *P. aeruginosa* were mixed at a 1:1 ratio, we observed an ~86% decrease in conjugation efficiency into a T6SS⁺ strain as compared with its isogenic T6SS⁻ *vipA* mutant (Fig. 3A). This reduction in transfer efficiency did not match the observed magnitude of killing of RP4⁺ MC1061 (Fig. 1A), probably because of intrinsic differences in the ability of

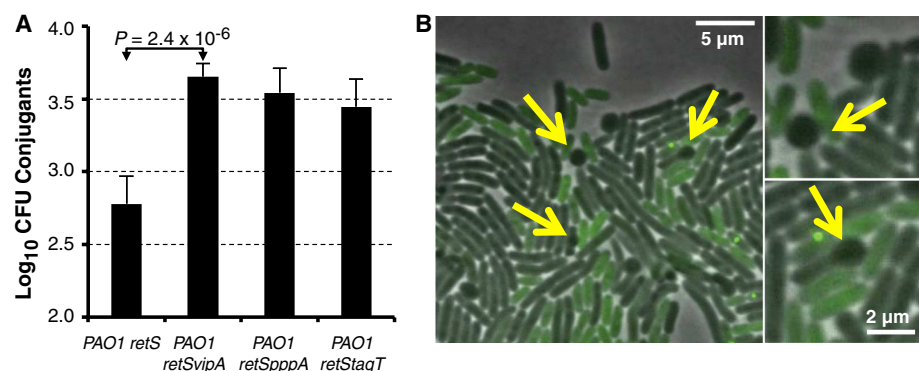


Fig. 3. Donor-directed T6SS attack blocks heterologous transfer of DNA. (A) The conjugation efficiency into different *P. aeruginosa* mutants. Data are mean $\pm$ SD, $n = 7$ independent replicates. (B) Representative field of cells containing a mixture of *P. aeruginosa* PAO1 $\Delta retS$ *clpV1-gfp* (green) and *E. coli* S17-1 RP4⁺ donor cells (nonfluorescent). *E. coli* cells exhibit cell rounding characteristic of T6SS-mediated killing (arrows). Larger magnification of rounding cells are shown in the insets.

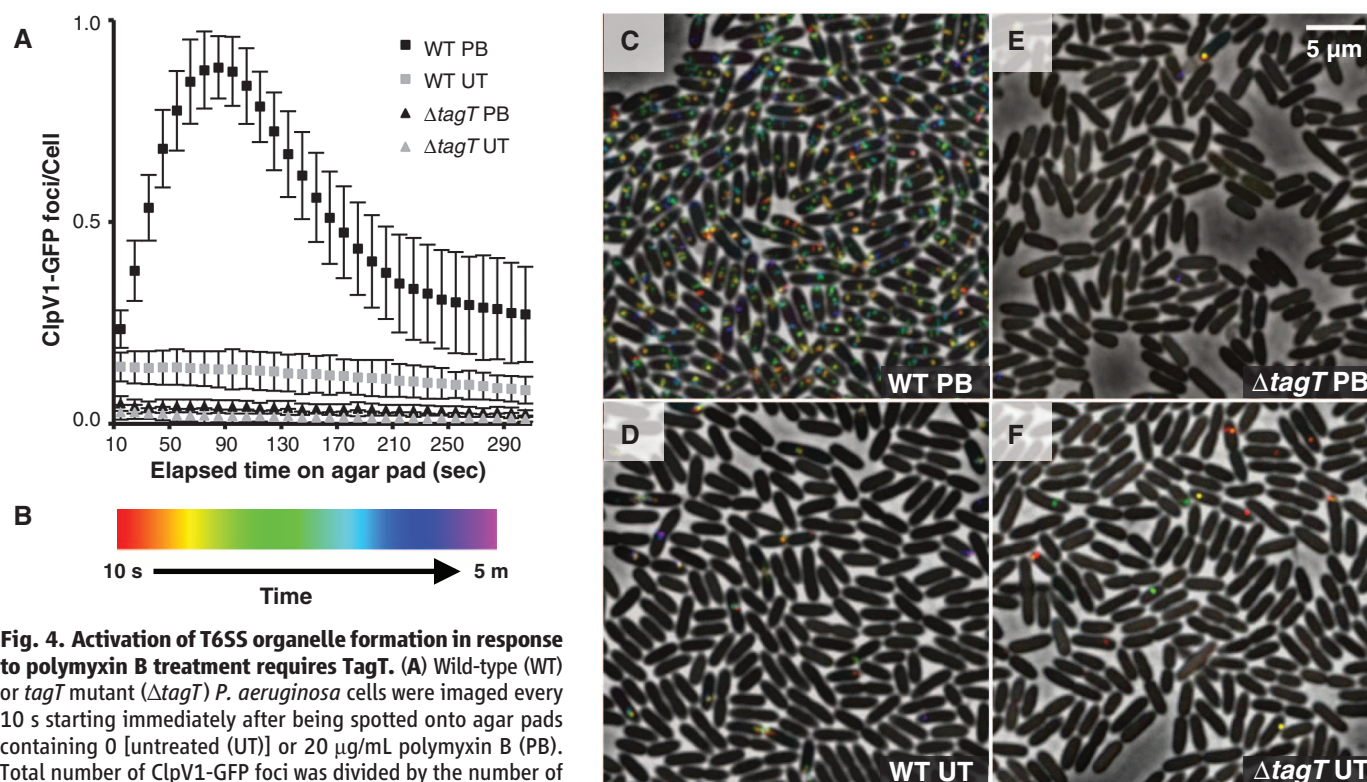


Fig. 4. Activation of T6SS organelle formation in response to polymyxin B treatment requires TagT. (A) Wild-type (WT) or *tagT* mutant ($\Delta tagT$) *P. aeruginosa* cells were imaged every 10 s starting immediately after being spotted onto agar pads containing 0 [untreated (UT)] or 20 μ g/mL polymyxin B (PB). Total number of ClpV1-GFP foci was divided by the number of cells for each field of cells to determine the average number of foci per cell. Each curve represents the mean of 12 to 16 fields with 250 to 600 cells in each field $\pm$ SD. (B) Color scale used to temporal-color code ClpV1-GFP signal. (C to F) ClpV1-GFP localization was followed for 5 min and temporally color-coded.

various donor strains to promote Mpf and T6SS activation with *P. aeruginosa*, which is similar to what we observed in our RP4 mutant analysis (Fig. 1D). *P. aeruginosa* mutants defective in the attack-sensing pathway genes *tagT* and *pppA* (10) also exhibited greater conjugation efficiency as recipient strains (Fig. 3A). Examination of mixtures of T6SS⁺ *P. aeruginosa* and *E. coli* RP4⁺ donor cells by means of fluorescence microscopy revealed rounding and blebbing of *E. coli* cells—a response that is typical of T6SS-mediated bacterial killing (Fig. 3B). Thus, inhibition of the conjugative transfer of pPSV35 was likely due to killing of *E. coli* cells through a donor-directed T6SS attack by *P. aeruginosa*.

The fact that multiple secretion systems can induce a T6SS counterattack suggested that the signal initiating this response really is a generalized disruption of the *P. aeruginosa* membrane. Accordingly, we asked whether polymyxin B—an antibiotic known to disrupt Gram-negative bacterial membranes by binding the lipid A component of lipopolysaccharides (24–26)—could induce T6SS activity in *P. aeruginosa*. We used a *P. aeruginosa* strain carrying a ClpV1-GFP and fluorescent time-lapse microscopy to monitor T6SS organelle formation and dynamics (9, 10) after exposure to polymyxin B. Cells exhibited a sixfold increase in the average number of visible ClpV1-GFP foci per cell within 90 s of being spotted onto agar pads containing 20 µg/mL of polymyxin B (Fig. 4, A and C, and movie S1). After this increase in T6SS activity, most ClpV1-GFP foci disappeared over the next 3 min, with the remaining foci becoming nondynamic (Fig. 4A and movie S1). The loss of dynamics likely reflects consumption of intracellular adenosine 5'-triphosphate pools after prolonged exposure to polymyxin B intoxication. This increase in T6SS activity was not observed when cells were spotted onto agar pads lacking polymyxin B (Fig. 4, A and D, and movie S1). Additionally, this increase in ClpV1-GFP foci was not observed in *tagT* mutants even in the presence of polymyxin B (Fig. 4, A, E, and F; and movie S2), suggesting that the same attack-sensing pathway that senses T4SS and T6SS attacks is responding to this antibiotic and mediates activation of the T6SS.

These studies support a model in which the donor-directed T6SS attack response in *P. aeruginosa* likely involves detection of perturbations in the cell envelope caused by the invasive components of the T4SS conjugation machinery. T6SS may represent a type of bacterial “innate immune system” that can detect and attack invading infectious elements not by recognizing their molecular patterns [such as nucleic acid sequences, as do the clustered regularly interspaced short palindromic repeat (CRISPR) elements (27, 28); or unmethylated DNA, as do restriction enzymes (29)] but rather by recognizing “transfer-associated patterns” (TAPs), including membrane disruptions that occur during interactions with other cells deploying T6SS and

T4SS translocation machines. Broad-host-range conjugative elements represent infectious bacterial “diseases” that can cause metabolic stress on their newly acquired hosts and thus represent a fitness burden to bacterial populations unable to combat their acquisition. The donor-directed T6SS attack paradigm may represent a strategy for suppressing the movement of horizontally transferred genetic elements in bacterial populations regardless of their signature molecular patterns (such as nucleic acid chemical structures or primary sequences).

References and Notes

- M. E. Hibbing, C. Fuqua, M. R. Parsek, S. B. Peterson, *Nat. Rev. Microbiol.* **8**, 15–25 (2010).
- S. Pukatzki et al., *Proc. Natl. Acad. Sci. U.S.A.* **103**, 1528–1533 (2006).
- F. Boyer, G. Fichant, J. Berthod, Y. Vandenbrouck, I. Attree, *BMC Genomics* **10**, 104 (2009).
- P. G. Leiman et al., *Proc. Natl. Acad. Sci. U.S.A.* **106**, 4154–4159 (2009).
- M. Basler, M. Pilhofer, G. P. Henderson, G. J. Jensen, J. J. Mekalanos, *Nature* **483**, 182–186 (2012).
- T. G. Dong, B. T. Ho, D. R. Yoder-Himes, J. J. Mekalanos, *Proc. Natl. Acad. Sci. U.S.A.* **110**, 2623–2628 (2013).
- R. D. Hood et al., *Cell Host Microbe* **7**, 25–37 (2010).
- J. D. Mougous et al., *Science* **312**, 1526–1530 (2006).
- M. Basler, J. J. Mekalanos, *Science* **337**, 815 (2012).
- M. Basler, B. T. Ho, J. J. Mekalanos, *Cell* **152**, 884–894 (2013).
- Z. Q. Luo, R. R. Isberg, *Proc. Natl. Acad. Sci. U.S.A.* **101**, 841–846 (2004).
- W. Pansegrau et al., *J. Mol. Biol.* **239**, 623–663 (1994).
- P. J. Christie, J. P. Vogel, *Trends Microbiol.* **8**, 354–360 (2000).
- G. Schröder, E. Lanka, *Plasmid* **54**, 1–25 (2005).
- L. A. Giebelhaus et al., *J. Bacteriol.* **178**, 6378–6381 (1996).
- Z. Li, H. Hiasa, U. Kumar, R. J. DiGate, *J. Biol. Chem.* **272**, 19582–19587 (1997).
- J. Haase, R. Lurz, A. M. Grahn, D. H. Bamford, E. Lanka, *J. Bacteriol.* **177**, 4779–4791 (1995).
- D. Fernandez, G. M. Spudich, X. R. Zhou, P. J. Christie, *J. Bacteriol.* **178**, 3168–3176 (1996).
- M. Bayer et al., *J. Bacteriol.* **177**, 4279–4288 (1995).
- P. J. Langer, W. G. Shanabruch, G. C. Walker, *J. Bacteriol.* **145**, 1310–1316 (1981).
- Z. Zhong, D. Helinski, A. Toukdarian, *Plasmid* **54**, 48–56 (2005).
- A. Rietsch, I. Vallet-Gely, S. L. Dove, J. J. Mekalanos, E. Exs, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 8006–8011 (2005).
- R. Simon, U. Priefer, A. Pühler, *Nat. Biotechnol.* **1**, 784–791 (1983).
- D. C. Morrison, D. M. Jacobs, *Immunochimistry* **13**, 813–818 (1976).
- J. B. McPhee, S. Lewenza, R. E. Hancock, *Mol. Microbiol.* **50**, 205–217 (2003).
- J. V. Hankins, J. A. Madsen, D. K. Giles, J. S. Brodbelt, M. S. Trent, *Proc. Natl. Acad. Sci. U.S.A.* **109**, 8722–8727 (2012).
- R. Barrangou et al., *Science* **315**, 1709–1712 (2007).
- P. Horvath, R. Barrangou, *Science* **327**, 167–170 (2010).
- T. Naito, K. Kusano, I. Kobayashi, *Science* **267**, 897–899 (1995).

Acknowledgments: Supporting movies and table can be found in the supplementary materials. This work was supported by National Institute of Allergy and Infectious Diseases grants AI-018045 and AI-26289 to J.J.M.

Supplementary Materials

www.sciencemag.org/content/342/6155/250/suppl/DC1
Materials and Methods

Table S1
References (30, 31)
Movies S1 and S2

25 July 2013; accepted 6 September 2013
10.1126/science.1243745

An Erythroid Enhancer of *BCL11A* Subject to Genetic Variation Determines Fetal Hemoglobin Level

Daniel E. Bauer,^{1,2,3} Sophia C. Kamran,^{3,4} Samuel Lessard,⁵ Jian Xu,^{1,3} Yuko Fujiwara,¹ Carrie Lin,¹ Zhen Shao,¹ Matthew C. Canver,³ Elenoe C. Smith,¹ Luca Pinello,⁶ Peter J. Sabo,^{7,8} Jeff Vierstra,^{7,8} Richard A. Voit,⁹ Guo-Cheng Yuan,^{6,10} Matthew H. Porteus,⁹ John A. Stamatoyannopoulos,^{7,8} Guillaume Lettre,⁵ Stuart H. Orkin^{1,2,3,4*}

Genome-wide association studies (GWASs) have ascertained numerous trait-associated common genetic variants, frequently localized to regulatory DNA. We found that common genetic variation at *BCL11A* associated with fetal hemoglobin (HbF) level lies in noncoding sequences decorated by an erythroid enhancer chromatin signature. Fine-mapping uncovers a motif-disrupting common variant associated with reduced transcription factor (TF) binding, modestly diminished *BCL11A* expression, and elevated HbF. The surrounding sequences function in vivo as a developmental stage-specific, lineage-restricted enhancer. Genome engineering reveals the enhancer is required in erythroid but not B-lymphoid cells for *BCL11A* expression. These findings illustrate how GWASs may expose functional variants of modest impact within causal elements essential for appropriate gene expression. We propose the GWAS-marked *BCL11A* enhancer represents an attractive target for therapeutic genome engineering for the β -hemoglobinopathies.

Genome-wide association studies (GWASs) have identified numerous common single-nucleotide polymorphisms (SNPs) associated with human traits and diseases. However, advancing from genetic association to causal bi-

ologic process has been challenging (1). Recent genome-scale chromatin mapping studies have highlighted the enrichment of GWAS variants in regulatory DNA elements, suggesting many causal variants may affect gene regulation (2–6). GWASs

of HbF level have identified trait-associated variants at *BCL11A* (supplementary text) (7–12). The transcriptional repressor BCL11A has been validated as a direct regulator of HbF level (13–18). Although constitutive BCL11A deficiency results in embryonic lethality and impaired lymphocyte development (19, 20), erythroid-specific deficiency of BCL11A counteracts developmental silencing of embryonic and fetal globin genes and rescues the hematologic and pathologic features of sickle cell disease (SCD) in mouse models (17).

¹Division of Hematology/Oncology, Boston Children's Hospital, Boston, MA 02115, USA. ²Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA. ³Harvard Medical School, Boston, MA 02115, USA. ⁴Howard Hughes Medical Institute, Boston, MA 02115, USA. ⁵Montreal Heart Institute and Université Montréal, Montreal, Quebec H1T 1C8, Canada. ⁶Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, MA 02115, USA. ⁷Department of Genome Sciences, University of Washington, Seattle, WA 98195, USA. ⁸Department of Medicine, University of Washington, Seattle, WA 98195, USA. ⁹Department of Pediatrics, Stanford University, Palo Alto, CA 94304, USA. ¹⁰Harvard School of Public Health, Boston, MA 02115, USA.

*Corresponding author. E-mail: stuart_orkin@dfci.harvard.edu

To further understand how common genetic variation affects *BCL11A*, HbF level, and β -globin disorder severity, we compared the distribution of the HbF-associated SNPs at *BCL11A* with deoxyribonuclease I (DNase I) sensitivity, which is an indicator of chromatin state suggestive of regulatory potential. In primary human erythroblasts, three peaks of DNase I hypersensitivity were observed in intron-2, adjacent to and overlying the HbF-associated variants (Fig. 1A). We term these DNase I hypersensitive sites (DHSs) +62, +58, and +55 based on distance in kilobases from the transcription start site (TSS) of *BCL11A*. Brain and B-lymphocytes, two tissues that express high levels, and T-lymphocytes, which do not express BCL11A, showed distinct patterns of DNase I sensitivity at the *BCL11A* locus, with a paucity of hypersensitivity overlying the trait-associated SNPs (Fig. 1A and fig. S1).

Chromatin immunoprecipitation sequencing (ChIP-seq) demonstrated histone modifications with an enhancer signature overlying the trait-associated SNPs at *BCL11A* intron-2, including the presence of H3K4me1 and H3K27ac and absence of H3K4me3 and H3K27me3 marks

(Fig. 1A and fig. S1). The major erythroid TFs GATA1 and TAL1 also occupy this enhancer region. ChIP-quantitative polymerase chain reaction (PCR) confirmed three discrete peaks of GATA1 and TAL1 binding within *BCL11A* intron-2, each falling within an erythroid DHS (Fig. 1B). A common feature of distal regulatory elements is long-range interaction with cognate promoters. We evaluated the interactions between the *BCL11A* promoter and fragments across 250 kb of the *BCL11A* locus using a chromosome conformation capture assay. The greatest promoter interaction was observed within the region of intron-2 containing the trait-associated SNPs (Fig. 1C).

We hypothesized that the causal trait-associated SNPs could function by modulating critical cis-regulatory elements. Therefore, we performed extensive genotyping of SNPs within the three erythroid DHSs +62, +58, and +55 in 1263 DNA samples from the Cooperative Study of SCD (CSSCD) (21). We used 1178 individuals and 38 SNPs for association testing (fig. S2A). Analysis of common variants [minor allele frequency (MAF) > 1%] revealed that rs1427407 in DHS +62 had the strongest association to HbF

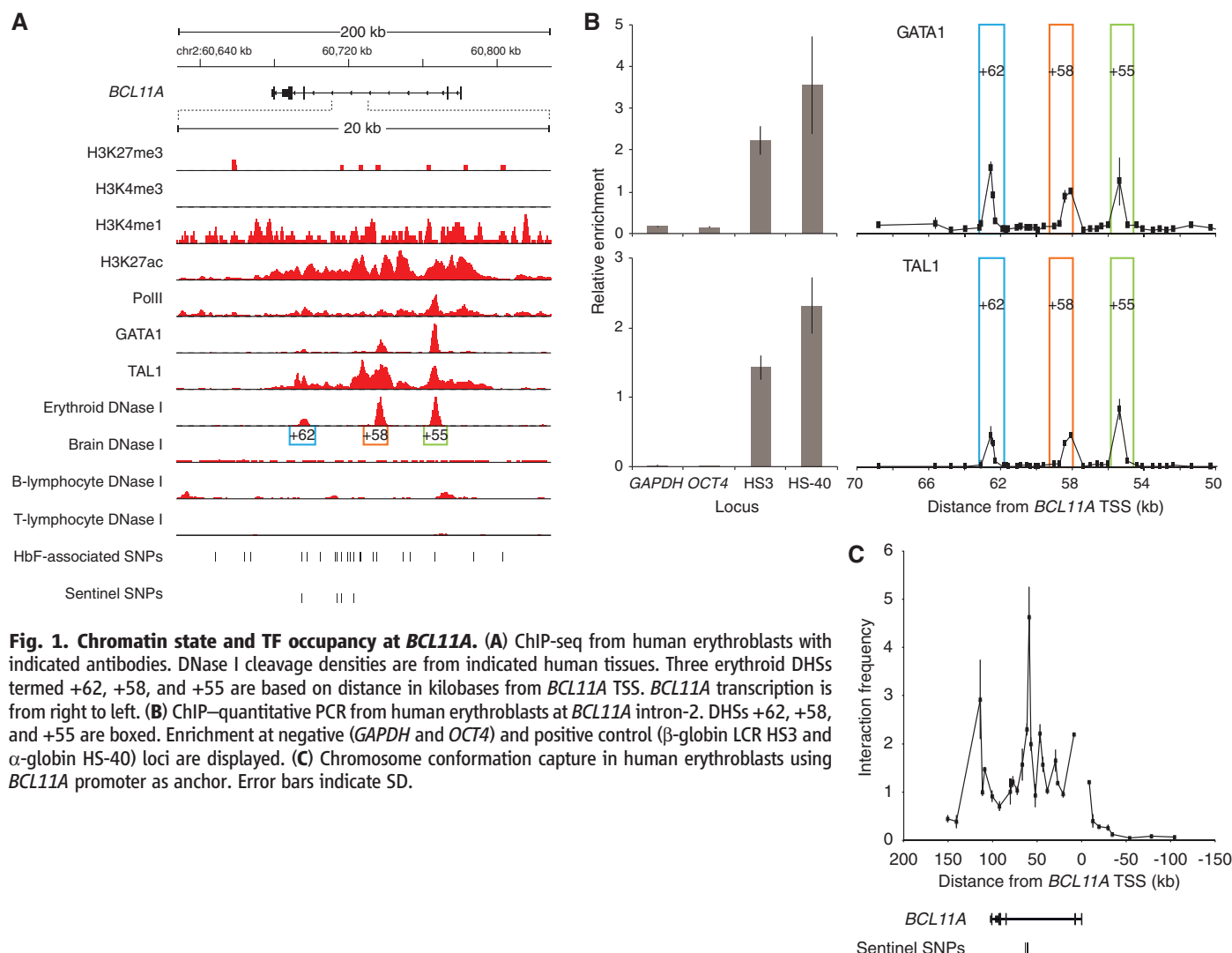


Fig. 1. Chromatin state and TF occupancy at *BCL11A*. (A) ChIP-seq from human erythroblasts with indicated antibodies. DNase I cleavage densities are from indicated human tissues. Three erythroid DHSs termed +62, +58, and +55 are based on distance in kilobases from *BCL11A* TSS. *BCL11A* transcription is from right to left. (B) ChIP-quantitative PCR from human erythroblasts at *BCL11A* intron-2. DHSs +62, +58, and +55 are boxed. Enrichment at negative (*GAPDH* and *OCT4*) and positive control (β -globin LCR HS3 and α -globin HS-40) loci are displayed. (C) Chromosome conformation capture in human erythroblasts using *BCL11A* promoter as anchor. Error bars indicate SD.

Fig. 2. Regulatory variants at *BCL11A*. (A) Genotype data obtained in 1178 individuals from CSSCD for 38 variants within *BCL11A* +62, +58, or +55 DHSs. Shown are most highly significant associations to HbF level among common (MAF > 1%) SNPs ($n = 10$ variants) before (rs1427407) or after (rs7606173) conditional analysis on rs1427407. SNP coordinates are chromosome 2, build hg19. (B) Chromatin from erythroblasts of individuals heterozygous for rs1427407, immunoprecipitated by GATA1 or TAL1 and pyrosequenced to quantify the relative abundance of the rs1427407-G allele. Composite half E-box–GATA motif previously identified (23) is shown. (C) gDNA and cDNA from erythroblasts of individuals heterozygous for rs1427407, rs7606173, and rs7569946. Haplotyping demonstrated rs7569946-G, rs1427407-G, and rs7606173-C on the same chromosome in each. Pyrosequencing was performed to quantify the relative abundance of the rs7569946-G allele.

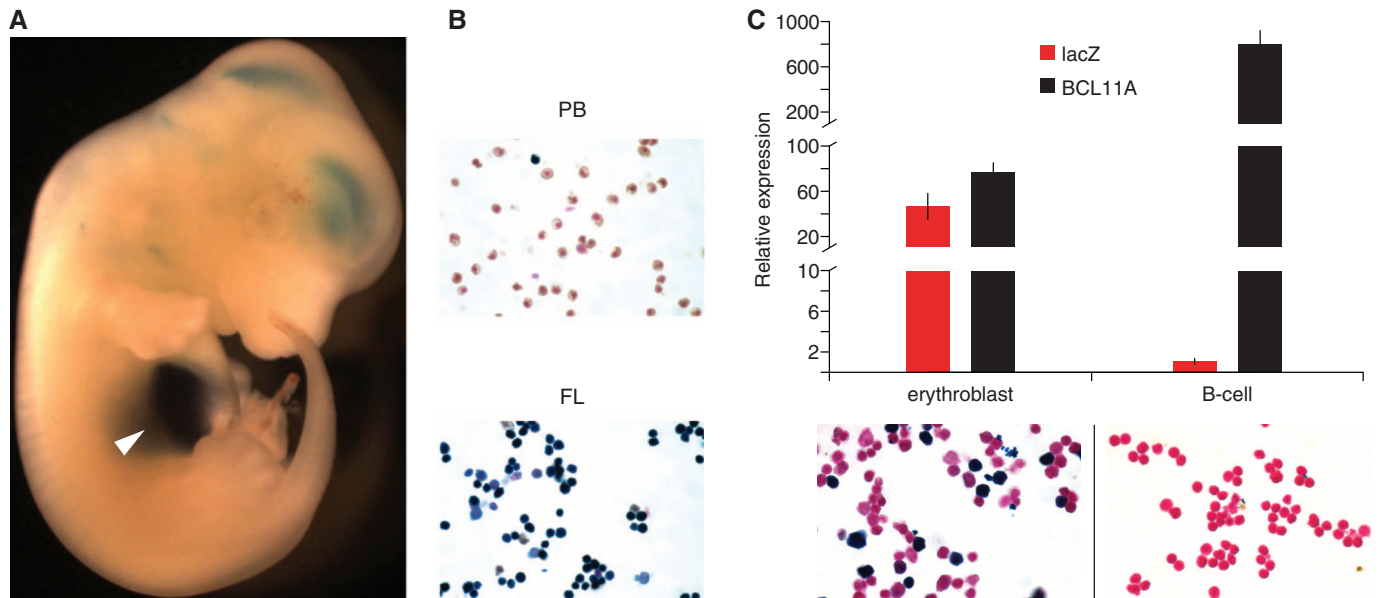
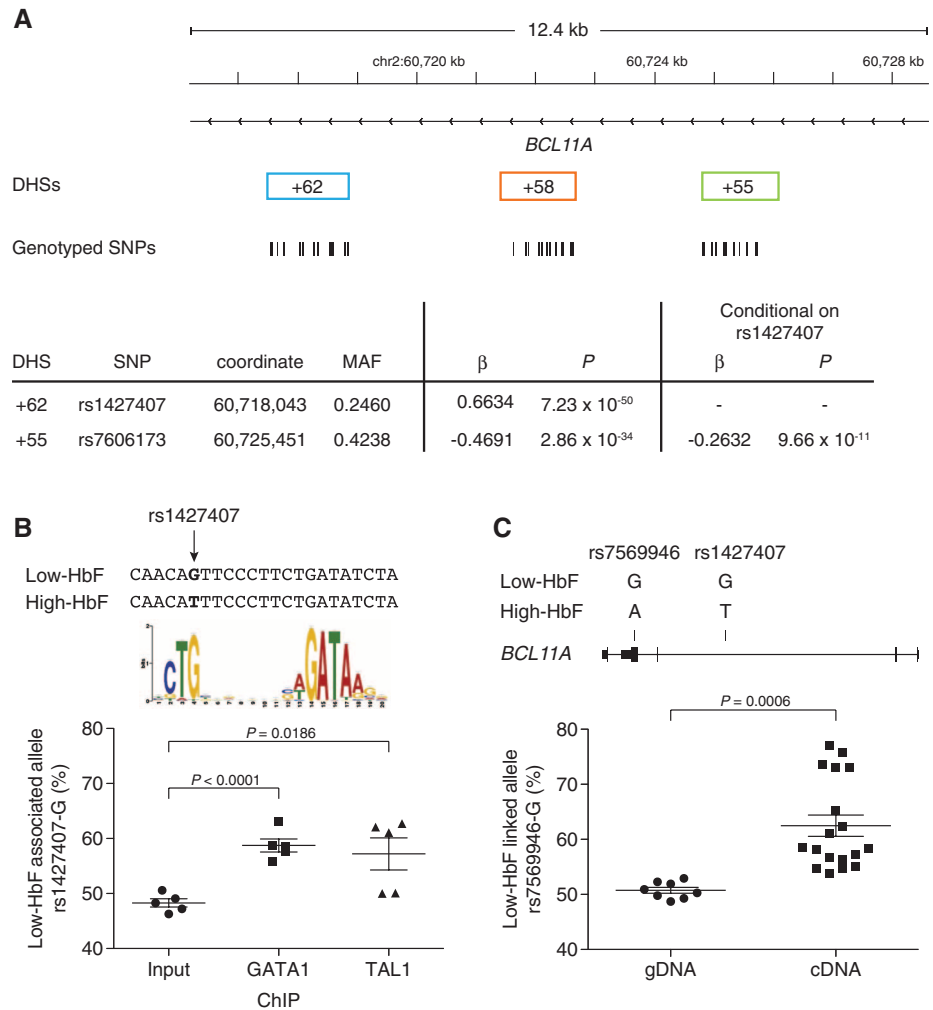


Fig. 3. The GWAS-marked *BCL11A* enhancer is sufficient for adult-stage erythroid expression. (A) A 12.4-kb fragment of *BCL11A* intron-2 (+52.0 to 64.4 kb from TSS) was cloned to a *lacZ* reporter construct. Shown is a transient transgenic mouse embryo from 12.5 dpc X-gal stained. Arrowhead indicates liver. (B) Cell suspensions isolated from peripheral blood (PB) and fetal liver

(FL) of stable transgenic embryos at 12.5 dpc X-gal stained. (C) Sorted erythroblasts and B-lymphocytes from young adult stable transgenic mice subject to X-gal staining or RNA isolation followed by quantitative reverse transcription (RT)-PCR. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and expressed relative to T-lymphocytes. Error bars indicate SD.

level ($P = 7.23 \times 10^{-50}$) (Fig. 2A, fig. S2B, and supplementary text). We identified associations to HbF level within the three DHSs that remained after conditioning on rs1427407 (Fig. 2A and fig. S2B), which is consistent with the hypothesis that multiple functional SNPs within the composite enhancer act combinatorially to influence BCL11A regulation. The most significant residual association was for rs7606173 in DHS +55 ($P = 9.66 \times 10^{-11}$).

The SNP rs1427407 falls within a peak of GATA1 and TAL1 binding (Fig. 1, A and B). The minor T-allele disrupts the G-nucleotide of a sequence element resembling a half E-box/GATA composite motif [CTG(n_9)GATA], a consensus sequence enriched for chromatin bound by GATA1 and TAL1 complexes in erythroid cells (22, 23). We identified five primary erythroblast samples from individuals heterozygous for the major G-allele and minor T-allele at rs1427407 and subjected these samples to ChIP followed by pyrosequencing. As anticipated, we observed an even balance of alleles in the input DNA. However, we detected more frequent binding to the G-allele as compared with the T-allele in both the GATA1 and TAL1 immunoprecipitated chromatin samples (Fig. 2B).

Because the common synonymous SNP rs7569946 lies within exon-4 of *BCL11A*, it can be used to discriminate expression of alleles. We identified three primary erythroblast samples doubly heterozygous for the rs1427407–rs7606173 haplotype and rs7569946. For each sample, we determined by means of molecular haplotyping that the major rs7569946 G-allele was in phase with the low-HbF-associated rs1427407–rs7606173 G–C haplotype (table S4) (24, 25). Pyrosequencing revealed that whereas the alleles were balanced in genomic DNA (gDNA), significant imbalance was observed in complementary DNA (cDNA) with 1.7-fold increased expression of the low-HbF-linked G-allele of rs7569946 (Fig. 2C and supplementary text).

To understand the context within which these apparent regulatory trait-associated SNPs play their role, we explored the function of the harboring composite element. We cloned a 12.4-kb (+52.0 to 64.4 kb from TSS) human gDNA fragment containing the three erythroid DHSs in order to assay enhancer potential in a mouse transgenic *lacZ* reporter assay (fig. S4). Endogenous BCL11A shows abundant expression throughout the developing central nervous system, with much lower expression observed in the fetal liver (26). In contrast, we observed in the transgenic embryos reporter gene expression largely confined to the fetal liver, the site of definitive erythropoiesis, with weaker expression noted in the central nervous system (Fig. 3A).

A characteristic feature of globin gene and *BCL11A* expression is developmental regulation (supplementary text). In stable transgenic *BCL11A* +52.0- to 64.4-kb reporter lines at 12.5 days post coitum (dpc), circulating primitive erythrocytes failed to stain for X-gal, whereas definitive erythroblasts in fetal liver robustly stained positive (Fig. 3B). Endogenous BCL11A was expressed at 10.4-fold-higher levels in B-lymphocytes as compared with erythroblasts. LacZ expression was restricted to erythroblasts and not observed in B-lymphocytes (Fig. 3C). These results indicate that the GWAS-marked *BCL11A* intron-2 regulatory sequences are sufficient to specify developmentally restricted, erythroid-specific gene expression.

We aimed to disrupt the enhancer to investigate its requirement for BCL11A expression. Because there are no suitable adult-stage human erythroid cell lines, we turned to the mouse erythroleukemia (MEL) cell line. We observed an orthologous enhancer signature at intron-2 of mouse *Bcl11a* indicated by sequence homology, erythroid-specific DNase I hypersensitivity, characteristic histone marks, and GATA1/TAL1 occupancy (fig. S6) (22, 27). Sequence-specific nucleases can produce small chromosomal deletions via nonhomologous

end joining (NHEJ)-mediated repair (28). We engineered transcription activator-like effector nucleases (TALENs) to introduce double-strand breaks to flank the orthologous 10-kb *Bcl11a* intron-2 sequences carrying the erythroid enhancer chromatin signature (fig. S7A). Three different clones were isolated that had undergone biallelic excision of the intronic segment (figs. S7 and S8 and supplementary text). BCL11A transcript was profoundly reduced in the absence of the orthologous erythroid composite enhancer (Fig. 4A). BCL11A protein expression was not detectable in the enhancer-deleted clones (Fig. 4B). In the absence of the BCL11A enhancer, embryonic globin gene derepression was pronounced, with the ratio of embryonic $\epsilon\gamma$ to adult $\beta 1/2$ globin increased by a mean of 364-fold (fig. S9).

To examine potential lineage-restriction of the requirement for the +50.4- to 60.4-kb intronic sequences for BCL11A expression, we evaluated their loss in a nonerythroid context. The same strategy of introduction of two pairs of TALENs to obtain clones with NHEJ-mediated deletion was used in a pre-B lymphocyte cell line. In contrast to the erythroid cells, BCL11A expression was retained in the $\Delta 50.4$ - to 60.4-kb enhancer deleted pre-B cell clones at both the RNA and protein levels (Fig. 4, A and B). These results indicate that the orthologous erythroid enhancer sequences are essential for erythroid gene expression but are not required in B-lymphoid cells for integrity of transcription from the *Bcl11a* locus.

The prior identification of BCL11A as a critical repressor of HbF levels has raised new hope for mechanism-based therapeutic approaches to the β -hemoglobinopathies (29). However, the paradox that genetic variation at *BCL11A* is common, well-tolerated, and disease-protective despite the critical roles of BCL11A in neurogenesis and lymphopoiesis (19, 20, 30) has remained unresolved. We have demonstrated that the HbF-associated variants localize to an erythroid enhancer of *BCL11A*. Through allele-specific analyses, we show that

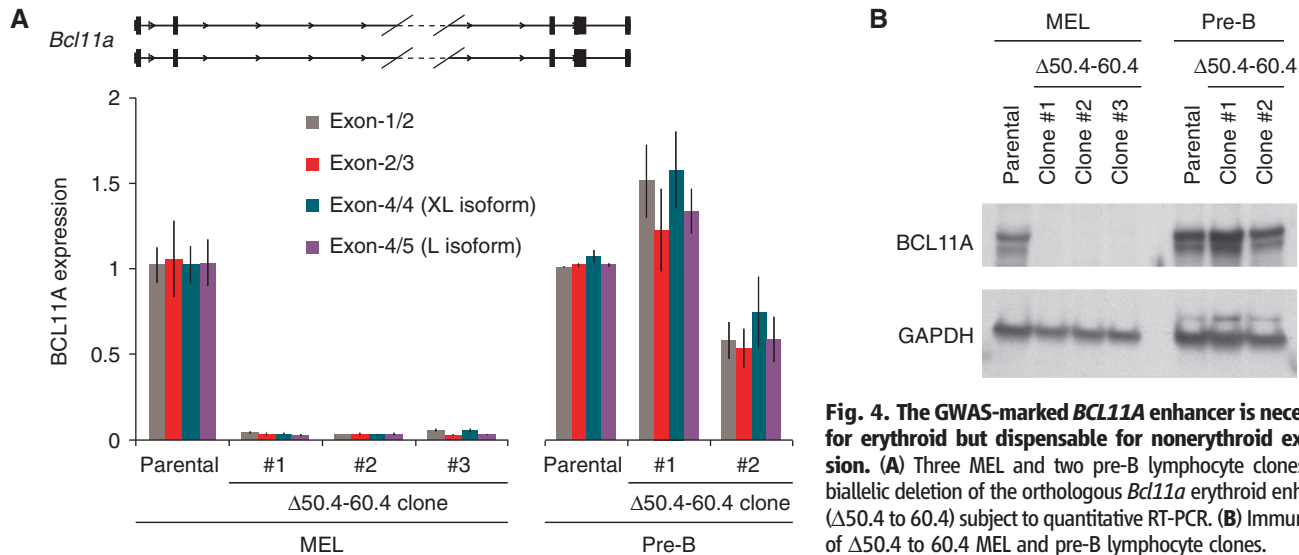


Fig. 4. The GWAS-marked *BCL11A* enhancer is necessary for erythroid but dispensable for nonerythroid expression. (A) Three MEL and two pre-B lymphocyte clones with biallelic deletion of the orthologous *Bcl11a* erythroid enhancer ($\Delta 50.4$ to 60.4) subject to quantitative RT-PCR. (B) Immunoblot of $\Delta 50.4$ to 60.4 MEL and pre-B lymphocyte clones.

genetic variation within this enhancer is associated with modest impact on TF binding, BCL11A expression, and HbF level. Relatively small effect sizes associated with individual variants may not be surprising given that most single-nucleotide substitutions, even within critical motifs, result in only modest loss of enhancer activity (31, 32). In contrast, loss of the *BCL11A* enhancer results in the absence of BCL11A expression in the erythroid lineage. Most trait-associated SNPs identified by GWAS are noncoding and have small effect sizes (1, 33). The impact of GWAS-identified SNPs on biological processes is often uncertain. Our findings underscore how a modest influence engendered by an individual noncoding variant neither predicts nor precludes a profound contribution of an underlying regulatory element.

Challenges to inhibiting BCL11A for mechanism-based reactivation of HbF include the supposedly “undruggable” nature of transcription factors (34) and its important nonerythroid functions (20, 30). With recent developments in their efficiency and precision, sequence-specific nucleases can be designed to exquisitely target genomic sequences of interest (35–37). We propose the GWAS-identified enhancer of *BCL11A* as a particularly promising therapeutic target for genome engineering in the β -hemoglobinopathies. Disruption of this enhancer would impair BCL11A expression in erythroid precursors with resultant HbF derepression while sparing BCL11A expression in nonerythroid lineages. Rational intervention might mimic common protective genetic variation.

References and Notes

- L. Fugger, G. McVean, J. I. Bell, *N. Engl. J. Med.* **367**, 2370–2371 (2012).
- J. Ernst et al., *Nature* **473**, 43–49 (2011).
- D. S. Paul et al., *PLoS Genet.* **7**, e1002139 (2011).
- M. T. Maurano et al., *Science* **337**, 1190–1195 (2012).
- P. van der Harst et al., *Nature* **492**, 369–375 (2012).
- ENCODE Project Consortium et al., *Nature* **489**, 57–74 (2012).
- S. Menzel et al., *Nat. Genet.* **39**, 1197–1199 (2007).
- M. Uda et al., *Proc. Natl. Acad. Sci. U.S.A.* **105**, 1620–1625 (2008).
- G. Lettre et al., *Proc. Natl. Acad. Sci. U.S.A.* **105**, 11869–11874 (2008).
- M. Nuinon et al., *Hum. Genet.* **127**, 303–314 (2010).
- N. Solovieff et al., *Blood* **115**, 1815–1822 (2010).
- P. Bhatnagar et al., *J. Hum. Genet.* **56**, 316–323 (2011).
- V. G. Sankaran et al., *Science* **322**, 1839–1842 (2008).
- J. Xu et al., *Genes Dev.* **24**, 783–798 (2010).
- J. Xu et al., *Proc. Natl. Acad. Sci. U.S.A.* **110**, 6518–6523 (2013).
- V. G. Sankaran et al., *Nature* **460**, 1093–1097 (2009).
- J. Xu et al., *Science* **334**, 993–996 (2011).
- F. Esteghamat et al., *Blood* **121**, 2553–2562 (2013).
- P. Liu et al., *Nat. Immunol.* **4**, 525–532 (2003).
- Y. Yu et al., *J. Exp. Med.* **209**, 2467–2483 (2012).
- M. D. Farber, M. Koshy, T. R. Kinney, The Cooperative Study of Sickle Cell Disease, *J. Chronic Dis.* **38**, 495–505 (1985).
- E. Soler et al., *Genes Dev.* **24**, 277–289 (2010).
- M. T. Kassouf et al., *Genome Res.* **20**, 1064–1083 (2010).
- D. J. Turner, M. E. Hurler, *Nat. Protoc.* **4**, 1771–1783 (2009).
- J. Tyson, J. A. Armour, *BMC Genomics* **13**, 693 (2012).
- M. Leid et al., *Gene Expr. Patterns* **4**, 733–739 (2004).
- M. S. Kowalczyk et al., *Mol. Cell* **45**, 447–458 (2012).
- H. J. Lee, E. Kim, J. S. Kim, *Genome Res.* **20**, 81–89 (2010).
- D. E. Bauer, S. C. Kamran, S. H. Orkin, *Blood* **120**, 2945–2953 (2012).
- A. John et al., *Development* **139**, 1831–1841 (2012).
- R. P. Patwardhan et al., *Nat. Biotechnol.* **30**, 265–270 (2012).
- A. Melnikov et al., *Nat. Biotechnol.* **30**, 271–277 (2012).
- K. A. Frazer, S. S. Murray, N. J. Schork, E. J. Topol, *Nat. Rev. Genet.* **10**, 241–251 (2009).
- A. N. Koehler, *Curr. Opin. Chem. Biol.* **14**, 331–340 (2010).
- F. D. Urnov, E. J. Rebar, M. C. Holmes, H. S. Zhang, P. D. Gregory, *Nat. Rev. Genet.* **11**, 636–646 (2010).
- J. K. Joung, J. D. Sander, *Nat. Rev. Mol. Cell Biol.* **14**, 49–55 (2013).
- J. van der Oost, *Science* **339**, 768–770 (2013).

Acknowledgments: We thank A. Woo, A. Cantor, M. Kowalczyk, S. Burns, J. Wright, J. Snow, J. Trowbridge, and members of the Orkin laboratory—particularly C. Peng, P. Das, G. Guo, M. Kerenyi, and E. Baena—for discussions. C. Guo and F. Alt provided the pre-B cell line; A. He and W. Pu provided the pWHERE lacZ reporter construct; C. Currie and M. Nguyen provided technical assistance; D. Bates and T. Kutayin provided expertise with sequence analysis; R. Sandstrom provided help with data management; G. Losyev and J. Daley provided aid with flow cytometry; and J. Desimini provided graphical assistance. L. Yan at EpigenDx (Hopkinton, Massachusetts) conducted the custom pyrosequencing reactions. This work was funded by grants from the Doris Duke Charitable Foundation (2009089) and Canadian Institute of Health Research (123382) to G.L.; Amon Carter Foundation, Hyundai Hope on Wheels, NIH, Lucille Packard Foundation to M.H.P.; NIH grants U54HG004594 and U54HG007010 to J.A.S.; and NIH R01HL032259, P01HL032262, and P30DK049216 (Center of Excellence in Molecular Hematology) to S.H.O. D.E.B. is supported by National Institute of Diabetes and Digestive and Kidney Diseases Career Development Award K08DK093705. D.E.B., J.X., and S.H.O. are inventors on a patent application related to this work, filed by Boston Children’s Hospital. The CSSCD samples with DNA and associated phenotype information are available from the National Heart, Lung, and Blood Institute to researchers that have appropriate institutional review board approval to use the materials.

Supplementary Materials

www.sciencemag.org/content/342/6155/253/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S9
Tables S1 to S6
References (38–54)

18 June 2013; accepted 13 September 2013
10.1126/science.1242088

Ancient DNA Reveals Key Stages in the Formation of Central European Mitochondrial Genetic Diversity

Guido Brandt,^{1*†} Wolfgang Haak,^{2*†} Christina J. Adler,³ Christina Roth,¹ Anna Szécsényi-Nagy,¹ Sarah Karimnia,¹ Sabine Möller-Rieker,¹ Harald Meller,⁴ Robert Ganslmeier,⁴ Susanne Friederich,⁴ Veit Dresely,⁴ Nicole Nicklisch,¹ Joseph K. Pickrell,⁵ Frank Sirocko,⁶ David Reich,⁵ Alan Cooper,^{2,†} Kurt W. Alt,^{1,†} The Genographic Consortium[§]

The processes that shaped modern European mitochondrial DNA (mtDNA) variation remain unclear. The initial peopling by Palaeolithic hunter-gatherers ~42,000 years ago and the immigration of Neolithic farmers into Europe ~8000 years ago appear to have played important roles but do not explain present-day mtDNA diversity. We generated mtDNA profiles of 364 individuals from prehistoric cultures in Central Europe to perform a chronological study, spanning the Early Neolithic to the Early Bronze Age (5500 to 1550 calibrated years before the common era). We used this transect through time to identify four marked shifts in genetic composition during the Neolithic period, revealing a key role for Late Neolithic cultures in shaping modern Central European genetic diversity.

The Central European Neolithic and the subsequent Early Bronze Age (EBA) reflect periods of momentous cultural changes (1–4). However, the extent to which such prehistoric cultural changes were accompanied by differences in the underlying genetics of local populations (1–5) and how such population shifts contributed to the present-day genetic diversity of Central Europe (6–9) are yet to be understood. Ancient DNA studies have revealed genetic discontinuities between indigenous hunter-gatherers and early farmers and between the latter and present-day Europeans (10, 11). Although this

confirms the importance of genetic shifts after the arrival of farming, the number and sequence of events and their potential origins and contributions to the genetic composition of modern-day Central Europe remain unclear (5, 6, 12).

We collected samples from 25 sites of the Mittelbe-Saale region in Saxony-Anhalt, Germany, attributed to nine archaeological cultures of the Early, Middle, and Late Neolithic period and the EBA, spanning ~4000 years (Fig. 1A, figs. S1 and S2, and table S1) (13). Mittelbe-Saale played a key role in human prehistory in Central Europe (4, 13), and the continuous settlement activity

from the Palaeolithic until today provides a detailed record of Neolithic cultures, including those with expansive European importance, such as the Linear Pottery (LBK), Funnel Beaker (FBC),

Corded Ware (CWC), and Bell-Beaker cultures (BBC) (fig. S2) (1–4, 13). We genotyped the hypervariable segment I and II of the control region and 22 single-nucleotide coding region polymorphisms from 364 individuals (tables S2 and S3) (13), allowing unambiguous haplogroup assignment, in order to characterize changes in the mitochondrial DNA (mtDNA) variability of the Mittelbe-Saale cultures. To examine genetic affinities of the investigated cultures to prehistoric and modern-day populations, we used 198 mtDNA data from published Mesolithic, Neolithic, and Bronze Age specimens across western Eurasia (Fig. 1B and table S4) (13) and a database of 67,996 sequences from present-day Eurasian populations (13). We animated our results to illustrate the observed changes in space and time (movie S1).

In order to detect patterns of continuity or discontinuity among and between the archaeological cultures, we conducted a cluster analysis

(Fig. 2A and table S5) based on haplogroup frequencies and used sequence data to perform a genetic distance analysis (F_{st}) (Fig. 2, B and C, and table S6) and analyses of molecular variance (AMOVA) (table S7) (13). We performed a Mantel test to examine whether the genetic distances correlate with the temporal distances between the ancient cultures, as expected from genetic drift affecting small populations. However, the Mantel test shows no strong correlation with time (Pearson's coefficient $r = 0.3923$, $P = 0.0591$), suggesting more sudden and marked fluctuations in genetic composition. We also developed a test for population continuity (TPC) (Fig. 2D and table S8) to further evaluate whether changes in haplogroup frequencies and composition could be explained by genetic drift or are likely due to other factors such as introgression via migration (introducing new haplogroups) or replacement (13). Our detailed transect through time reveals

¹Institute of Anthropology, Johannes Gutenberg University of Mainz, Colonel-Kleinmann-Weg 2, D-55128 Mainz, Germany.
²The Australian Centre for Ancient DNA, University of Adelaide, Adelaide, South Australia 5005, Australia. ³Institute of Dental Research, Westmead Millennium Institute, Faculty of Dentistry, University of Sydney, Sydney, New South Wales 5006, Australia.
⁴State Office for Heritage Management and Archaeology Saxony-Anhalt and Heritage Museum, Richard-Wagner-Straße 9, D-06114 Halle (Saale), Germany. ⁵Department of Genetics, Harvard Medical School, Boston, MA 02115, USA. ⁶Institute of Geosciences, Johannes Gutenberg University of Mainz, Johann-Joachim-Becher-Weg 21, D-55128 Mainz, Germany.
 *Corresponding author. E-mail: brandtg@uni-mainz.de (G.B.); wolfgang.haak@adelaide.edu.au (W.H.)
 †These authors contributed equally to this work.
 ‡These authors contributed equally to this work.
 §Consortium members are listed in the supplementary materials.

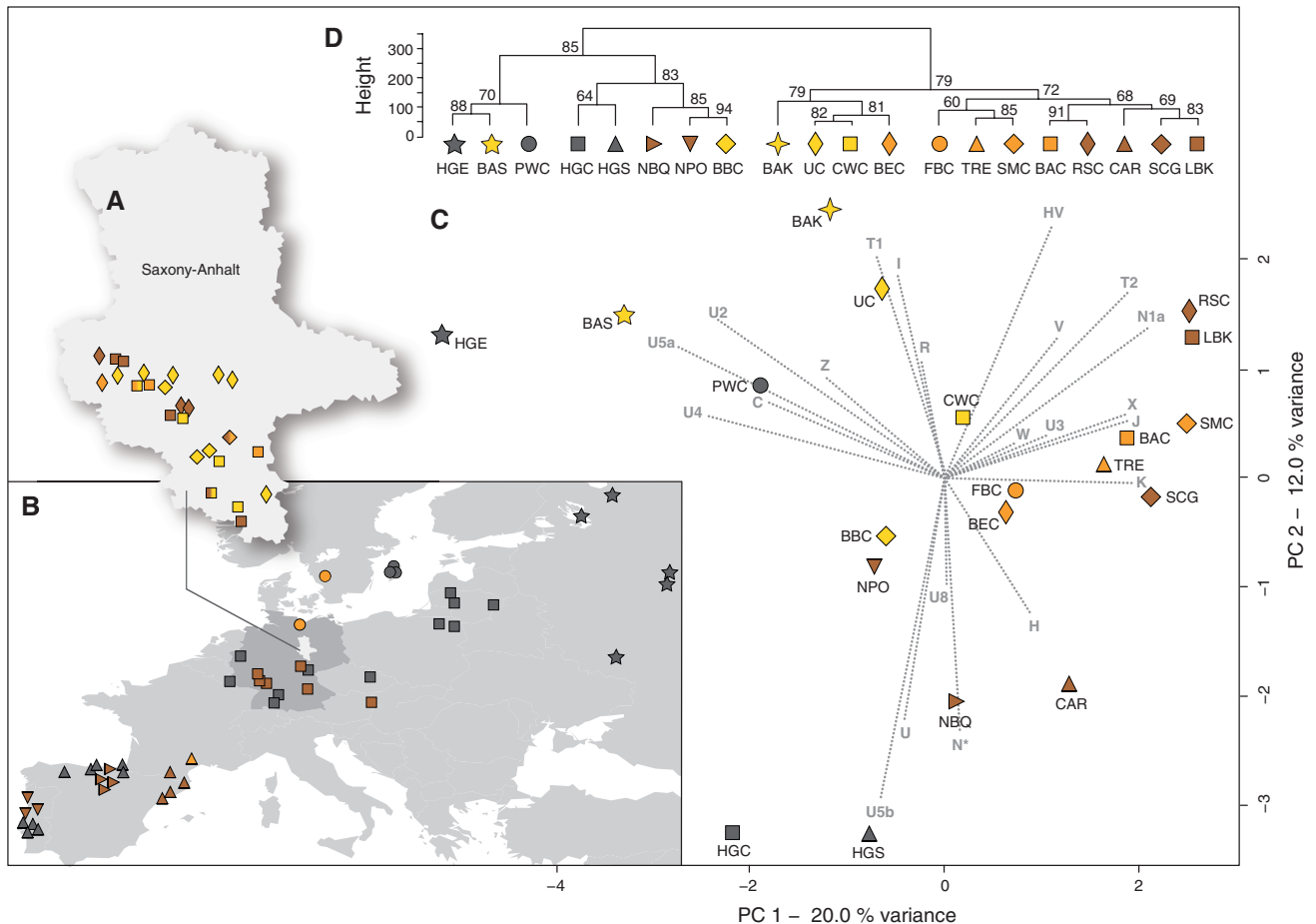


Fig. 1. Location of Mittelbe-Saale and prehistoric comparative data, as well as PCA and Ward clustering. (A) The locations of study sites in the Mittelbe-Saale region in Saxony-Anhalt, Germany, of the Early Neolithic (LBK; RSC, Rössen culture; and SCG, Schöningen group), Middle Neolithic (BAC, Baalberge culture), SMC, Salzmünde culture; and BEC), Late Neolithic (CWC and BBC), and Early Bronze Age (UC, Unetice culture) cultures. (B) The locations of published data from 11 Mesolithic (HGC, hunter-gatherer Central Europe; HGS, hunter-gatherer South Europe; HGE, hunter-gatherer East Europe; and PWC, Pitted Ware culture), Neolithic [CAR, (Epi)Cardial; NPO, Neolithic Portugal; NBQ, Neolithic Basque Country and Navarre; FBC; TRE, Treilles culture], and Bronze Age [BAS, Bronze Age Siberia; BAK, Bronze Age Kazakhstan

(not shown)] populations. Symbols indicate populations from Central Europe (squares and diamonds), southern Scandinavia (circles), the Iberian Peninsula (triangles), and East Europe/Asia (stars). Color shading of data points denote to hunter-gatherer (gray), Early Neolithic (brown), Middle Neolithic (orange), and Late Neolithic/EBA (yellow) samples [for further information see (13), figs. S1 and S2, and tables S1 to S4]. The haplogroup frequencies of these populations (table S9) were used to perform PCA (C) and Ward clustering (D). The first two principal components of the PCA display 32.8% of the total genetic variation. We superimposed each haplogroup as component loading vectors (gray), proportionally to their contribution. P values of the clusters are given in percent of reproduced clusters based on 10,000 bootstrap replicates.

a complex pattern of both genetic continuity and discontinuity (Fig. 2, A to D, and tables S5 to S8), based on the assumption that haplogroups are monophyletic and neutral, that is, not evolving into new haplogroups via mutations from an existing haplogroup or resulting from selection. Indigenous Central European hunter-gatherers (10, 14) are set apart from the Neolithic Mittelbe-Saale cultures on the basis of both cluster analysis (Fig. 2A) and significantly different F_{st} values ($F_{st} = 0.0845$ to 0.21358 , $P = 0.00000$ to 0.03292) (Fig. 2B), because of mutually exclusive haplogroup compositions (fig. S3 and movie S1). The results of the TPC show that the transition from hunter-gatherers to the LBK farmers cannot be explained by genetic drift alone ($P = 0.000001$) (Fig. 2D), consistent with previous findings (10, 11).

The Mittelbe-Saale cultures themselves can be further differentiated into distinct Early/Middle Neolithic and Late Neolithic/EBA clusters (Fig. 2A), as shown by significantly higher F_{st} values ($F_{st} = 0.02776$ to 0.05605 , $P = 0.00000$ to 0.016616) (Fig. 2, B and C). The two groupings are also strongly supported in AMOVA tests, where 289 different combinations of the ancient cultures were examined. We found the highest among-group variance, and low variation within the groups, when the Mittelbe-Saale cultures

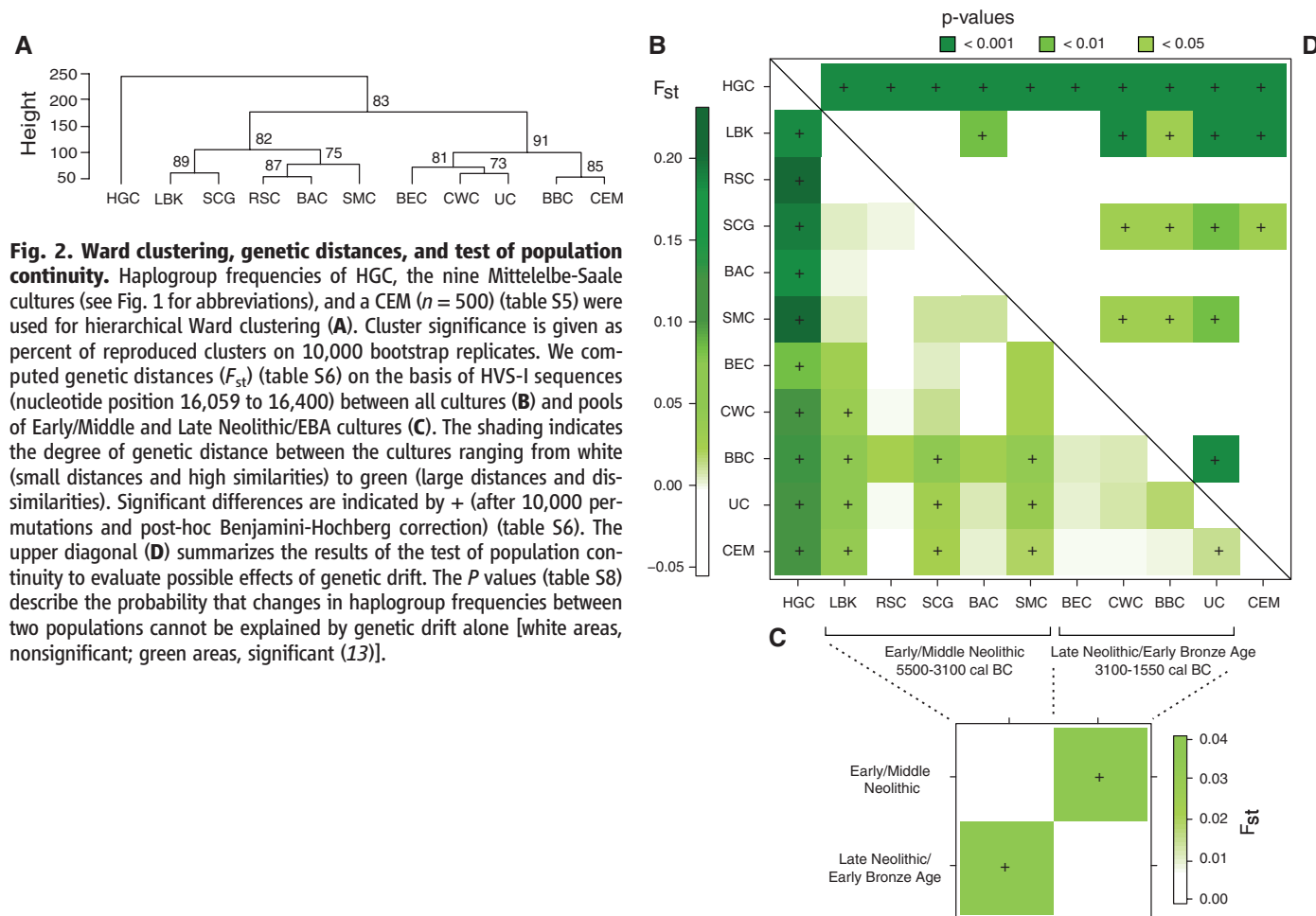
were separated into two groups of Early/Middle Neolithic and Late Neolithic/EBA cultures (among groups 3.06% , $F_{st} = 0.03061$, $P = 0.00683$; within groups 0.45% , $F_{st} = 0.00468$, $P = 0.18891$) (table S7). Similarly, TPC also indicates that changes in the mtDNA profiles between most of the Early/Middle Neolithic cultures and the Late Neolithic/EBA ($P = 0.000007$ to 0.049428) as well as between the BBC and EBA ($P = 0.000803$) (Fig. 2D) cannot be explained by drift alone. These results suggest multiple population genetic shifts: the first during the introduction of farming, followed by further changes during the later Neolithic.

To further explore these patterns, we used a principal component analysis (PCA) and a cluster analysis (Fig. 1, C and D, and table S9) to describe the characteristic haplogroups of each culture and to identify genetic affinities to other prehistoric populations (13). We then examined affinities to present-day Eurasian populations to inform on potential geographic origins of the different cultures. We performed multidimensional scaling (MDS) (fig. S4, A to I, and table S10) based on continuous sequence data, which is sensitive to shared haplotypes between populations (13). In parallel, we also used PCA (fig. S5, A to I, and table S11), Procrustes and cluster analyses (fig. S6, A to I, and table S12), and genetic dis-

tance mapping (fig. S7, A to I, and table S13) based on discrete haplogroup frequencies (13).

Detailed investigation of the mtDNA composition of each culture reveals a series of haplogroup frequency changes because of different genetic profiles for hunter-gatherers, the Early/Middle Neolithic group, and individual cultures of the later Neolithic/EBA including the Bernburg culture (BEC) and the temporally overlapping BBC, CWC, and EBA (Fig. 3, fig. S3, and movie S1). The latter suggests that this period was heterogeneous, with genetically differentiated cultures resulting in a separation in the PCA (Fig. 1C). These shifts are also visible in the genetic distance maps and Procrustes-projected PCAs, where the Near Eastern affinity of the LBK and its subsequent regional derivatives switches to a clear European affinity in later Neolithic/EBA cultures, with distinct geographic orientations (see below; movie S1; and figs. S6, A to I, and S7, A to I).

We synthesized the different lines of evidence from our comparative genetic analyses to reconstruct a series of four prominent population dynamic events (termed A to D; Fig. 3 and movie S1), which we reconcile with known European cultural expansions (1–5). Overall, these analyses reveal a pattern of relative genetic continuity for the first 2500 years after the introduction of farming



in Central Europe, followed by a series of discontinuities in the later Neolithic.

Event A marks the transition from foraging to farming introduced by the LBK, which reached Central Europe ~5500 calibrated years before the common era (cal BCE) (movie S1) (1–3). Mitochondrial data from Central European hunter-gatherers comprises exclusively U lineages (U, U4, U5, and U8) (10, 14), whereas the LBK is characterized by a distinct haplogroup profile including N1a, T2, K, J, HV, V, W, and X (Fig. 1C) (11). These haplogroups can be denoted as a mitochondrial “Neolithic package” and comprise around 79.4% of the diversity in the LBK, whereas hunter-gatherer lineages are rare (2.9%) (Fig. 3). This marked shift suggests a rapid transition process, with the comparative analyses indicating a genetic influx from the Near East, Anatolia, and the Caucasus (movie S1 and figs. S4A to S7A) (1–3, 11). The subsequent Early/Middle Neolithic cultures closely resemble the mtDNA haplogroup composition of the LBK (Figs. 1, C and D, and 2, A and D, and table S7) with similar affinities to present-day Near East populations (figs. S4, B to E, and S7, B to E), suggesting a period of genetic continuity over 2500 years.

Event B describes a bidirectional interaction along a north-south axis during the Early and Middle Neolithic, which saw the introduction of

the Neolithic package to southern Scandinavia by Central European cultures (B₁ ~4100 cal BCE), followed by a reflux of hunter-gatherer lineages to Central Europe (B₂ ~3100 cal BCE) (movie S1). The Neolithic transition of southern Scandinavia was closely linked to the FBC, which replaced local foragers that had retained the Mesolithic lifestyle for ~1500 years after farming arrived in Central Europe (1–3). FBC individuals from Scandinavia (10, 15, 16) have yielded high frequencies of hunter-gatherer haplogroups (30%) alongside a large amount of Neolithic package haplogroups (60%) (table S9), leading to an intermediate position between hunter-gatherers and the Early/Middle Neolithic Mittelbe-Saale cultures in the PCA (Fig. 1C). This suggests that pioneer groups from Central Europe had interacted with local hunter-gatherers who adopted farming (movie S1) (1–4), a view also supported by ancient genomic data (16). Subsequently, around a millennium later in Mittelbe-Saale, a genetic shift associated with the BEC (Fig. 1, A to D, and table S7), a late representative of the FBC in Central Europe (4), saw an increase in hunter-gatherer lineages (29.4%) and a decrease in farmer lineages (47.1%) (Fig. 3), resulting in a haplogroup composition similar to that of the Scandinavian FBC (Fig. 1C) (10, 15). Although previous populations show affinities to the Near

East, the BEC marks a clear shift toward those in present-day North Europe (movie S1 and figs. S4F to S7F).

In the Late Neolithic, we identify two independent events (C and D), each associated with major contemporary Pan-European phenomena. Event C (~2800 cal BCE) is marked by the emergence of the CWC (movie S1), whose subgroups were widespread across Central and Eastern Europe (fig. S2) (2–4). The CWC is characterized by haplogroups I and U2 (4.6%), which are new maternal elements in Mittelbe-Saale (Fig. 1C and fig. S3) and appear alongside other Late Neolithic/EBA lineages such as T1 (6.8%) and hunter-gatherer haplogroups U4 and U5 (20.5%), whereas Early/Middle Neolithic haplogroups further decrease (45.5%) (Fig. 3). The binomial probability that we missed I and U2 in 211 individuals of preceding cultures is very low ($P=0.00$). Haplogroup U2 has been reported exclusively from Paleolithic, Mesolithic, and Bronze Age samples from Russia (17–19), and PCA and cluster analyses reveal similarities of the CWC to two ancient Kurgan groups of South Siberia (19) and Kazakhstan (20) (Fig. 1, C and D), in which haplogroups I, U2, and T1 are frequent (18.2 to 37.5%) (table S9). Intriguingly, the Y chromosomal haplogroup R1a1a, frequent in ancient Siberian populations (19), has previously been detected in

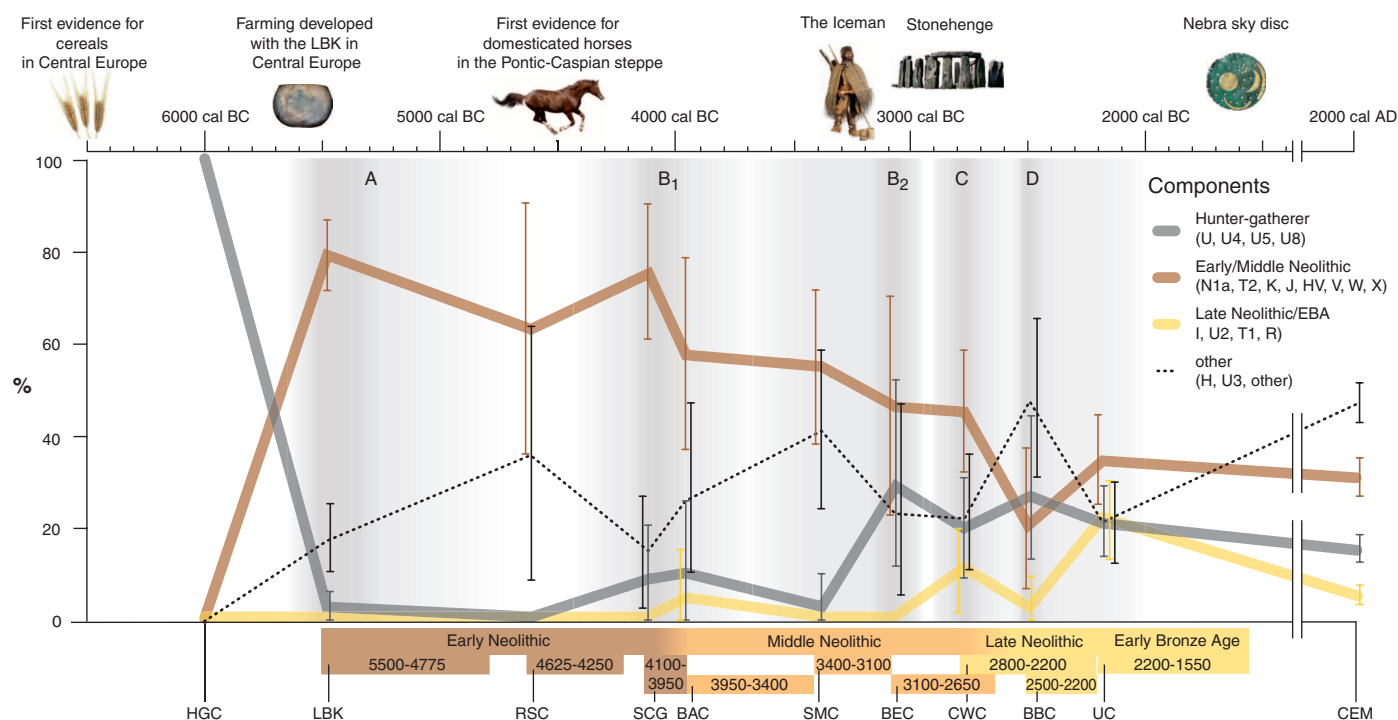
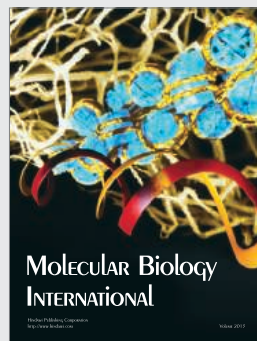
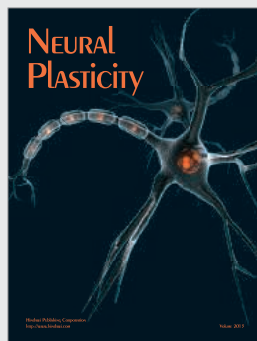
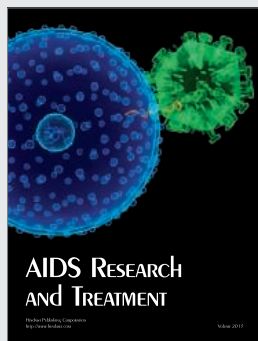
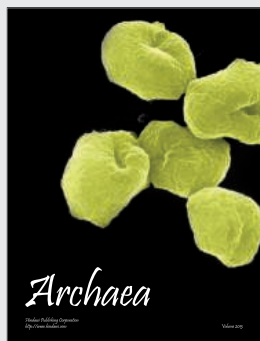
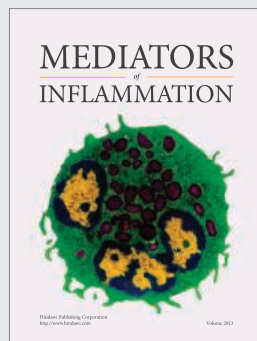
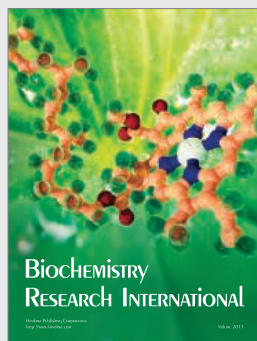
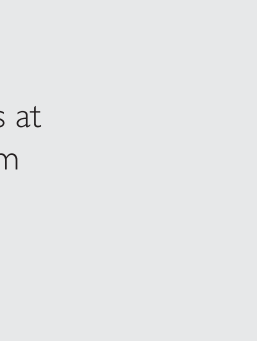
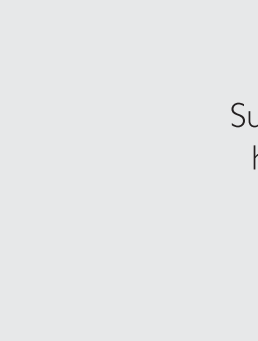
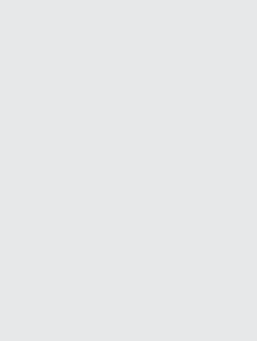
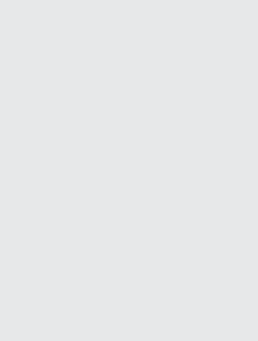


Fig. 3. Development of mtDNA components from the Late Mesolithic to present day. Population data from HGC, the nine Mittelbe-Saale cultures (see Fig. 1 for abbreviations), and a CEM ($n = 500$) were placed in chronological order (x axis), and the amounts of lineages ascribed to particular time periods were evaluated in each population. The characterizing haplogroups of the hunter-gatherer (U, U4, U5, and U8; gray), Early/Middle Neolithic (N1a, T2, K, J, HV, V, W, and X; brown), and Late Neolithic/EBA (I, U2, T1, and R; yellow) periods were summarized into three respective components (y axis)

(table S5) accordingly to the differentiation in the PCA (Fig. 1C). Haplogroups that could not be ascertained unambiguously to one of the three components were reported as “other” (H, U3, and other African and Asian lineages of the CEM) (13). Error bars of component frequencies indicate the 95% confidence interval of 10,000 bootstrap replicates (table S5). Horizontal shading denotes the population dynamic events (A, B₁, B₂, C, and D) inferred from the synthesis of all population genetic analyses (see main text).



Hindawi

Submit your manuscripts at
<http://www.hindawi.com>



2013 Laureate for Science
Harold Y. Hwang
Professor, Stanford University

2013 Laureate for Engineering
Sangtae Kim
Distinguished Professor, Purdue University

2013 Laureate for Medicine
Se-Jin Lee
Professor, Johns Hopkins University

The Ho-Am Prize ignites the passion and innovative spirit for scientific discovery

**We welcome your recommendations for the
2014 Ho-Am Prize candidates.**

- Award categories are Science, Engineering, and Medicine.
 - Ethnic Korean researchers are eligible for the Prize.
 - Deadline for submission is Nov. 30, 2013.
- *Learn more about candidate recommendation at www.hoamprize.org



To continue Samsung Founder "Ho-Am" Byung-Chull Lee's (1910-1987) efforts to maximize both human potential and public interest, Chairman Kun-Hee Lee of Samsung established the Prize in 1990. The Prize is awarded annually to recognize outstanding ethnic Korean researchers in Korea and around the world who have made important contributions to the advancement of science.

THE HO-AM PRIZE

Any sample, any application — no limits

**Maximize success
with QIAGEN sample technologies**

- Innovative, room-temperature sample collection and stabilization
- DNA, RNA, and protein purification from any sample
- Hands-free automated sample preparation
- Reliable genetic, epigenetic, and gene expression analysis from FFPE samples
- Whole genome and transcriptome amplification to overcome sample limitations

Contact QIAGEN today or visit www.qiagen.com/sample-technologies



Sample & Assay Technologies

Better results — on any sequencing platform

Get the most from your NGS

Discover new and innovative solutions,
dedicated for use with any NGS workflow



Streamline your next-generation sequencing (NGS) workflow and achieve high-quality results you can rely on.

- Highly specific and selective nucleic acid purification and target enrichment
- Unbiased whole genome amplification from a single cell
- High DNA library yields using optimized workflows that allow ~50% time-savings
- Easy-to-use, spin-column-based size selection of DNA for library construction
- Outstanding results on any sequencing platform

Visit www.qiagen.com/goto/NGS to learn more!



Sample & Assay Technologies

Make ends meet.



Gibson Assembly[®] Cloning Kit

New England Biolabs has revolutionized your laboratory's standard cloning methodology. The Gibson Assembly Cloning Kit combines the power of the Gibson Assembly Master Mix with NEB 5-alpha Competent *E. coli*, enabling fragment assembly and transformation in just under two hours. Save time, without sacrificing efficiency.

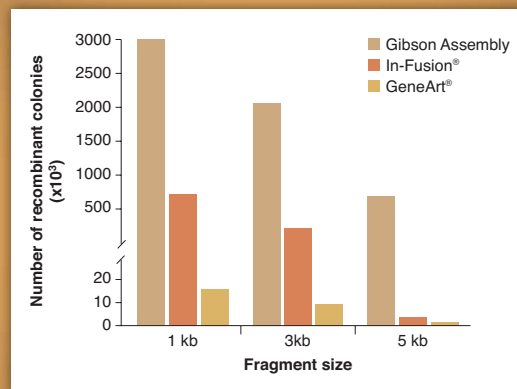
Making ends meet is now quicker and easier than ever before, with the Gibson Assembly Cloning Kit from NEB.

NEBuilder[™]
for Gibson Assembly

Visit NEBGibson.com to view the latest tutorials and to try our primer design tool.

IN-FUSION[®] is a registered trademark of Clontech Laboratories, Inc.
GENEART[®] is a registered trademark of Life Technologies, Inc.
GIBSON ASSEMBLY[®] is a registered trademark of Synthetic Genomics, Inc.

Gibson Assembly Cloning Kit provides robust transformation efficiencies



Assembly reactions containing 25 ng of linear pUC19 vector and 0.04 pmol of each fragment were performed following individual suppliers' recommended protocols and using the competent cells provided with the kit. The total number of recombinant colonies was calculated per 25 ng of linear pUC19 vector added to the assembly reaction.

SGIDNA

Some components of this product are manufactured by New England Biolabs, Inc. under license from Synthetic Genomics, Inc.

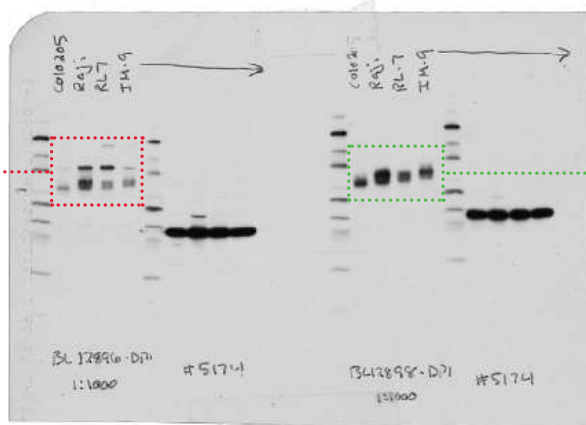
You've carefully
designed your
experiment.

Taylor Ngo started at CST 8
years ago and is currently in
the Molecular Biology group.

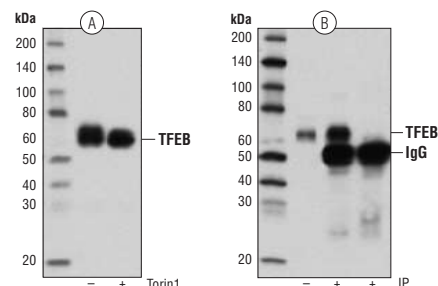
Does your antibody measure up?

We validate all our antibodies in-house. If it's not specific, it doesn't ship.

REJECTED
Extra Band
Weak Signal



**CST
APPROVED**
Clean Band
Strong Signal



TFEB Antibody #4240: (A) WB analysis of Raji cell extracts, untreated (-) or Torin1-treated, using #4240 (Torin1 treatment induces dephosphorylation). (B) IP of TFEB from COLO 205 cells using #4240 (lane 2) or Normal Rabbit IgG #2729 (lane 3). Lane 1 is 10% input.

WB analysis of various cell extracts using two development samples at 1:1000 dilution. GAPDH (D16H11) XP® Rabbit mAb #5174 was used as a loading control.

Please visit our website to request a copy of our new white paper – *A Guide to Successful Western Blotting*.

www.cellsignal.com/successfulWB

© 2013 Cell Signaling Technology, Inc. Cell Signaling Technology® and XP® are trademarks of Cell Signaling Technology, Inc.



Cell Signaling
TECHNOLOGY®

cOmplete ULTRA Tablets Ultra Efficient Protein Protection



Protect your precious proteins. Choose cOmplete ULTRA Tablets for effective protease inhibition.
Why settle for less. 8 powerful protease inhibitors in one tablet – more than in other protease inhibitor tablets.
Rely on Roche experience. cOmplete ULTRA builds on 18 years of proven performance worldwide.



Visit our new website for more information:
www.complete.roche.com

For life science research only.
Not for use in diagnostic procedures.
COMPLETE is a trademark of Roche.

Roche Diagnostics GmbH
Sandhofer Straße 116
68305 Mannheim, Germany

© 2013 Roche Diagnostics.
All rights reserved.



Take Control.

Design dynamic experiments the CellASIC™ way.

Biology is so much more than DMEM/FBS, 37 °C, 5% CO₂. Model experiments creatively and achieve true culture conditions with the CellASIC™ ONIX Microfluidic Platform. Push the boundaries of your cell biology experiments in an *in vivo*-like environment – it's easy to program automated changes to conditions and track cell responses with this flexible, intuitive platform.

The power is yours.
www.millipore.com/CellASIC



EMD Millipore is a division of Merck KGaA, Darmstadt, Germany

Confidence. Inspired by Results.

Alan Alfano
Cancer Researcher,
University of Maryland
School of Medicine,
Baltimore, MD

"In cancer research, achieving uniform results with transfection is often a big hurdle. **X-tremeGENE is by far the best transfection reagent I've ever used.** It's nice to know that with all the potential issues I might encounter in my research, transfection isn't one of them."

X-tremeGENE Reagents. Transfect with confidence.



See more of Alan's story at
www.x-tremegene.roche.com

For life science research only.
Not for use in diagnostic procedures.

X-TREMEGENE is a trademark of Roche.

Roche Diagnostics Corporation
Roche Applied Science
Indianapolis, Indiana

© 2013 Roche Diagnostics.
All rights reserved.

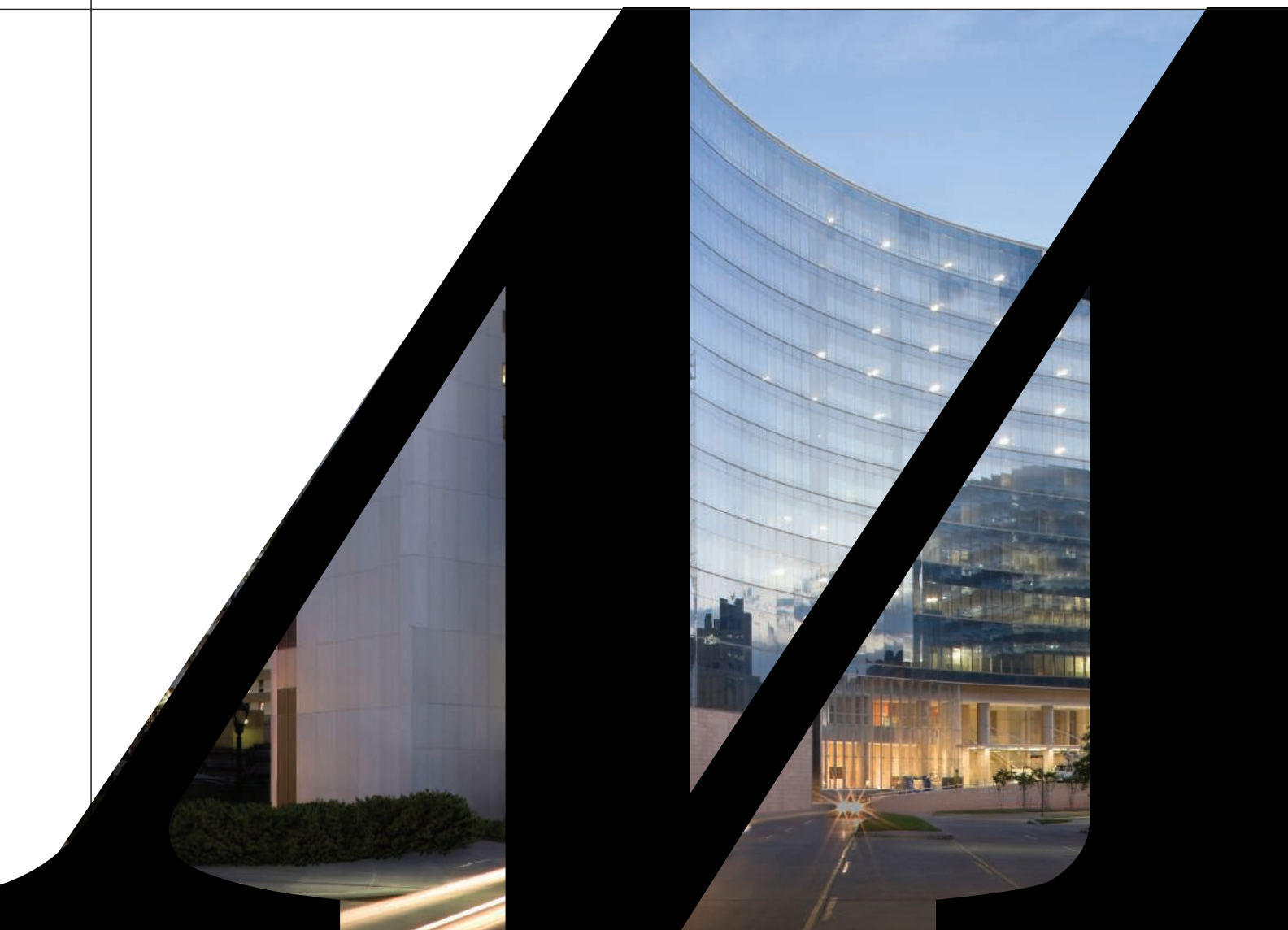


NEW NAME. NEW ADVANCES. THE METHODIST HOSPITAL IS NOW HOUSTON METHODIST HOSPITAL.

We've changed our name. The Methodist Hospital is now Houston Methodist Hospital. We're always moving forward, and we believe that our greatest advances are those that are just around the corner. So we continue to look toward the future, bringing the finest researchers and clinicians from around the world to Houston, to join us in the pursuit of better care and better cures. That's the difference between practicing medicine and leading it.

houstonmethodist.org

HOUSTON
MethodistSM
LEADING MEDICINE



EVERYONE DESERVES A CHANCE TO BREAK THROUGH

Ion Torrent™ next-generation sequencing platforms combine simple chemistry and proven semiconductor technology to provide accurate data at a low cost. Now, more labs can use sequencing in more ways than ever before.

ion torrent
Sequencing for all.™



TARGETED
SEQUENCING



EXOME
SEQUENCING



TRANSCRIPTOME
SEQUENCING



GENOME
SEQUENCING

Start sequencing now at lifetechnologies.com/iontorrent

For Research Use Only. Not for use in diagnostic procedures.
©2013 Life Technologies Corporation. All rights reserved. The trademarks mentioned herein are the property of Life Technologies Corporation and/or its affiliate(s) or their respective owners. C007023 0913



Visit the
Ion Bus at ASHG

[lifetechnologies.com/
ashg2013](http://lifetechnologies.com/ashg2013)

life
technologies™

Watch this ad
come to life!

Get the "digital PCR" app from
iTunes® or Google Play™, then point
your mobile device at this ad.



ABSOLUTELY ATTAINABLE

Introducing digital PCR for every lab—the simple, affordable,
absolute QuantStudio™ 3D Digital PCR System

Go beyond the limits of real-time PCR with the only chip-based digital PCR
system on the market. Never before has so much innovation, flexibility, and
precision been packed in an 8 x 5 x 9 inch instrument.



Go to lifetechnologies.com/quantstudio3d

life
technologies™

AAAS Travels



COPPER CANYON, MEXICO
April 10-17, 2014
Total Lunar Eclipse April 14-15, 2014!

Explore Copper Canyon or Barranca del Cobre on a memorable train adventure bringing you dramatic scenery, natural history and Tarahumara Indians, including the Total Lunar Eclipse over Copper Canyon!

The striking volcanic scenery, bridges, tunnels, and villages are seen from the "train in the sky," the Chihuahua al Pacifico. \$2,995 + air.

For a detailed brochure, please call (800) 252-4910

All prices are per person twin share + air



BETCHART EXPEDITIONS Inc.

17050 Montebello Rd, Cupertino, CA 95014

Email: AAASInfo@betchartexpeditions.com

www.betchartexpeditions.com



Join the Conversation!

Twitter is a great way to connect with AAAS members and staff about the issues that matter to you most. Be a part of the discussion while staying up-to-date on the latest news and information about your personal member benefits.

**Follow us @AAASmember
and join the conversation
with #AAAS**



MemberCentral.aaas.org



STAY INFORMED! STAY CONNECTED!

Get more from your
AAAS membership

Are you currently registered to receive e-mails from AAAS and *Science*?

E-mail is the primary way that AAAS communicates with our members about AAAS programs, new member benefits, invitations to special events, and, of course, the latest news and research being published in *Science*.

Sign up today to receive e-mails from AAAS and ensure that you are getting the most out of your membership and *Science* subscription.*

To get started visit:

promo.aaas.org/stayconnected

You'll need your AAAS Member number. Find it above your name on your *Science* mailing label.

Don't miss a thing. Sign up for e-mail communications from AAAS today!



*AAAS follows CAN-SPAM and European Safe Harbor guidelines for protecting your privacy. We will never sell your e-mail address and you can opt-out of receiving e-mails at any time.

ABSOLUTELY ATTAINABLE ONLINE SERVICE



Easily manage your
instrument use and care
on lifetechnologies.com*

Sign in to your account to:

- Access instrument service history
- Request service and get quotes
- Track warranty and contract status
- Check availability and schedule instrument use
- Discover many more benefits

No cost. No hassle. No worries.

Learn more at
[lifetechnologies.com/
easiertomanage](http://lifetechnologies.com/easiertomanage)



*At this time, Instrument Management is available in the United States, Canada, Australia, New Zealand, and most countries in Europe.
©2013 Life Technologies Corporation. All rights reserved. C007221 0913

Enhanced BD Horizon™ Brilliant Violet™ Reagents

New innovative formulation.



Revealing nature's secrets
with unprecedented clarity.

Now BD Horizon™ Brilliant Violet™ reagents have been enhanced for better performance. BD Biosciences commitment to quality includes continual improvement of the flow cytometry tools we produce. Those efforts have yielded BD Horizon Brilliant Violet polymer conjugates that can more easily and precisely help you resolve rare and dim cell populations. Now accompanied with an innovative BD Biosciences proprietary staining buffer, the combined formulation resolves possible dye-to-dye interactions for consistently predictable results.



Helping all people
live healthy lives

This pioneering polymer dye technology enables researchers to identify cell populations with lower receptor density than previously possible and resolve cell populations previously obscured. The complete portfolio of BD conjugated antibodies can be used to explore cellular features and characterize cells through surface and intracellular markers.

Request a free sample or find out how you can use BD reagents across your entire multicolor panel with an expanded set of tools and information at bdbiosciences.com/go/brilliant.



The New Legend

Eppendorf Reference® 2

»Reference« stands for extraordinary precision and accuracy, a long service life, and an ergonomic design. The new Reference 2 boasts these proven Premium characteristics and this operating philosophy with its innovative state-of-the-art technology; making it a reliable partner for you and your demanding work.

- > Single-button operation enables ergonomic handling with reduced operating effort
- > High precision and accuracy providing reliable pipetting results
- > Quick and secure volume setting, incl. volume lock
- > RFID chip contains all relevant data regarding the pipette



www.eppendorf.com/reference

Eppendorf®, the Eppendorf logo, Eppendorf PhysioCare Concept® and Eppendorf Reference® are registered Trademarks of Eppendorf AG, Germany. All right reserved, including graphics and images. Copyright © 2013 by Eppendorf AG.

SCIENCE & DIPLOMACY

A quarterly publication from the AAAS Center for Science Diplomacy

SCIENCE & DIPLOMACY provides an open access forum for rigorous thought, analysis, and insight to serve stakeholders who develop, implement, or teach all aspects of science and diplomacy.

WWW.SCIENCEDIPLOMACY.ORG



Register for free to receive regular updates:
www.sciencediplomacy.org/user/register

 @SciDip

Senior Advisory Board

Norman P. Neureiter (Chair), *AAAS*
Peter C. Agre, *Johns Hopkins*
David C. Clary, *Oxford*
Paula J. Dobriansky, *Harvard*
Esther Dyson, *EDventure*

Sumaya bint El Hassan, *Jordan's RSS*
Richard N. Foster, *Yale*
David A. Hamburg, *AAAS*
Mohamed Hassan, *IAP*
Neal F. Lane, *Rice*

Science & Diplomacy is published by the Center for Science Diplomacy of the American Association for the Advancement of Science (AAAS), the world's largest general scientific society.



Rethink Western blotting. Take protein detection to new dimensions.

Biology isn't flat—neither are your Westerns.

Meet the fast, versatile SNAP i.d.® 2.0 system, to fully exploit three-dimensional reagent distribution. Unlike conventional Western blotting, where diffusion is the primary means of reagent transport, the SNAP i.d.® 2.0 system applies a vacuum to actively drive antibodies and buffers right through the membrane. This advanced technology promotes antigen binding and thorough washing, enabling you to better optimize your Western blotting conditions.

www.emdmillipore.com/SNAP



EMD Millipore is a division of Merck KGaA, Darmstadt, Germany

EMD Millipore and the M mark are trademarks of Merck KGaA, Darmstadt, Germany. SNAP i.d. is a registered trademark of Merck KGaA, Darmstadt, Germany.
© 2013 EMD Millipore Corporation, Billerica, MA USA. All rights reserved. BS-GEN-13-07845.

AAAS|2014 ANNUAL MEETING

13-17 FEBRUARY • CHICAGO

MEETING GLOBAL CHALLENGES:
DISCOVERY AND INNOVATION

Advance your career at the 2014 AAAS Annual Meeting. Attend career workshops and scientific symposia, discuss your research, and network with thousands of leading scientists, engineers, educators, policymakers, and journalists.

Career Development Workshops include:

- Editing Your Own Papers and Proposals: How to Wow Reviewers and Aid Readers
- Sharing Science: Presenting Yourself and Your Work
- Grant Skills are Career Skills: Integrate to Accelerate
- Getting Started in Social Media
- Knowing Your Worth: The Art (and Etiquette) of Negotiating a Job Offer

The program schedule is now online.

Register today!

www.aaas.org/meetings

 #AAASmtg



2014 **MRS**

SPRING MEETING & EXHIBIT

April 21-25, San Francisco, CA



CALL FOR PAPERS

Abstract Deadline • November 1, 2013

Abstract Submission Site Opens • October 1, 2013

ENERGY

- A Film-Silicon Science and Technology
- B Organic and Inorganic Materials for Dye-Sensitized Solar Cells
- C Synthesis and Processing of Organic and Polymeric Materials for Semiconductor Applications
- D Materials for Photoelectrochemical and Photocatalytic Solar-Energy Harvesting and Storage
- E Earth-Abundant Inorganic Solar-Energy Conversion
- F Controlling the Interaction between Light and Semiconductor Nanostructures for Energy Applications
- G Photoactivated Chemical and Biochemical Processes on Semiconductor Surfaces
- H Defect Engineering in Thin-Film Photovoltaic Materials
- I Materials for Carbon Capture
- J Physics of Oxide Thin Films and Heterostructures
- K Nanostructures, Thin Films and Bulk Oxides—Synthesis, Characterization and Applications
- L Materials and Interfaces in Solid Oxide Fuel Cells
- M Fuel Cells, Electrolyzers and Other Electrochemical Energy Systems
- N Research Frontiers on Electrochemical Energy Storage Materials—Design, Synthesis, Characterization and Modeling
- O Novel Energy-Storage Technologies beyond Li-ion Batteries—From Materials Design to System Integration
- P Mechanics of Energy Storage and Conversion—Batteries, Thermoelectrics and Fuel Cells
- Q Materials, Technologies and Sensor Concepts for Advanced Battery Management Systems
- R Materials Challenges and Integration Strategies for Flexible Energy Devices and Systems
- S Actinides—Basic Science, Applications and Technology
- T Superconductor Materials—From Basic Science to Novel Technology

SOFT AND BIOMATERIALS

- U Soft Nanomaterials
- V Micro- and Nanofluidic Systems for Materials Synthesis, Device Assembly and Bioanalysis
- W Functional Biomaterials for Regenerative Engineering
- Y Biomaterials for Biomolecule Delivery and Understanding Cell-Niche Interactions
- Z Bioelectronics—Materials, Processes and Applications
- AA Advanced Multifunctional Biomaterials for Neuroprosthetic Interfaces

ELECTRONICS AND PHOTONICS

- BB Materials for End-of-Roadmap Devices in Logic, Power and Memory
- CC New Materials and Processes for Interconnects, Novel Memory and Advanced Display Technologies
- DD Silicon Carbide—Materials, Processing and Devices
- EE Advances in Inorganic Semiconductor Nanoparticles and Their Applications
- FF The Grand Challenges in Organic Electronics
- GG Few-Dopant Semiconductor Optoelectronics
- HH Phase-Change Materials for Memory, Reconfigurable Electronics and Cognitive Applications
- II Emerging Nanophotonic Materials and Devices
- JJ Materials and Processes for Nonlinear Optics
- KK Resonant Optics—Fundamentals and Applications
- LL Transparent Electrodes

NANOMATERIALS

- MM Nanotubes and Related Nanostructures
- NN 2D Materials and Devices beyond Graphene
- OO *De Novo* Graphene
- PP Nanodiamonds—Fundamentals and Applications
- QQ Computationally Enabled Discoveries in Synthesis, Structure and Properties of Nanoscale Materials
- RR Solution Synthesis of Inorganic Functional Materials
- SS Nanocrystal Growth via Oriented Attachment and Mesocrystal Formation
- TT Mesoscale Self-Assembly of Nanoparticles—Manufacturing, Functionalization, Assembly and Integration
- UU Semiconductor Nanowires—Synthesis, Properties and Applications
- VV Magnetic Nanomaterials and Nanostructures

GENERAL—THEORY AND CHARACTERIZATION

- WW Materials by Design—Merging Advanced *In-situ* Characterization with Predictive Simulation
- XX Shape Programmable Materials
- YY Meeting the Challenges of Understanding and Visualizing Mesoscale Phenomena
- ZZ Advanced Characterization Techniques for Ion-Beam-Induced Effects in Materials
- AAA Applications of *In-situ* Synchrotron Radiation Techniques in Nanomaterials Research
- BBB Advances in Scanning Probe Microscopy for Material Properties
- CCC *In-situ* Characterization of Material Synthesis and Properties at the Nanoscale with TEM
- DDD Atomic-Resolution Analytical Electron Microscopy of Disruptive and Energy-Related Materials
- EEE Materials Behavior under Extreme Irradiation, Stress or Temperature

SPECIAL SYMPOSIUM

- FFF Educating and Mentoring Young Materials Scientists for Career Development

www.mrs.org/spring2014

Meeting Chairs

Jose A. Garrido, Technische Universität München
Sergei V. Kalinin, Oak Ridge National Laboratory
Edson R. Leite, Federal University of Sao Carlos
David Parrillo, The Dow Chemical Company
Molly Stevens, Imperial College London

Don't Miss These Future MRS Meetings!

2014 MRS Fall Meeting & Exhibit
November 30-December 5, 2014

Hynes Convention Center & Sheraton Boston Hotel
Boston, Massachusetts

2015 MRS Spring Meeting & Exhibit
April 6-10, 2015

Moscone West & San Francisco Marriott Marquis
San Francisco, California



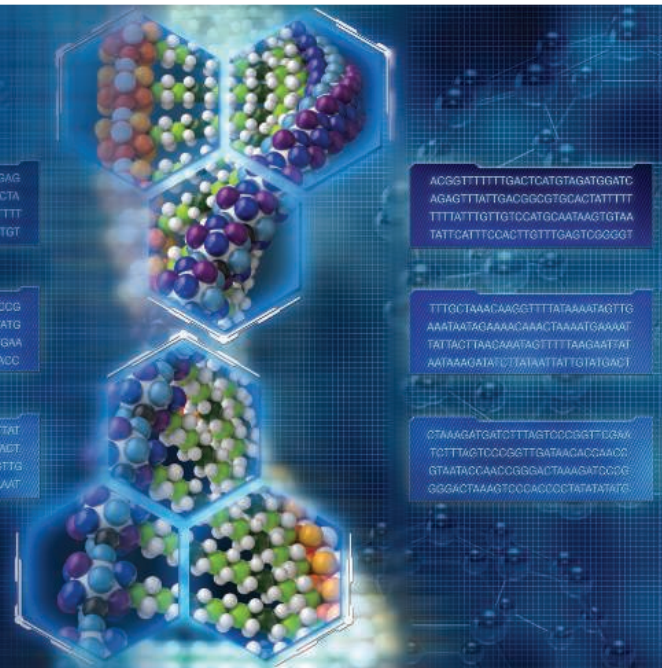
MATERIALS RESEARCH SOCIETY
Advancing materials. Improving the quality of life.

506 Keystone Drive • Warrendale, PA 15086-7573
Tel 724.779.3003 • Fax 724.779.8313
info@mrs.org • www.mrs.org

Exome Sequencing: Toward an Interpretable Genome

The U.S. National Human Genome Research Institute says it can sequence genomes for \$5,826 as of April 2013—just 0.006% of the \$95.2 million per-genome price tag in September 2001. Commercial entities can do it for even less, and by the end of 2013, the cost could well dip below \$1,000. Yet even at that low, low price, whole genome sequencing isn't cheap, at least not when researchers need to decode them by the thousands. Enter exome sequencing. Faster, cheaper, and more easily interpreted than its full-sized counterpart, an exome is to a genome as an abstract is to a research article: concise, information-rich, and easily digested.

By Jeffrey M. Perkel



“The exome right now is the part of the genome we know how to interpret.”

An exome is simply the protein-coding content of the genetic code, some 1%–2% of the genome in all. Since sequencers can read only so many bases per run, researchers sequencing exomes can produce more of them more quickly, at greater resolution and lower cost than they can whole genomes.

Joris Veltman, professor of translational genomics at **Radboud University Medical Center** in Nijmegen, the Netherlands, uses **Life Technologies'** SOLiD instruments for both research and clinical sequencing applications. His lab has the capacity to sequence a couple thousand exomes annually, he estimates, but just 50 or so whole genomes. “Throughput is ... a major reason why we choose [to do] exomes,” he says.

Plus, there's interpretability. Whole-genome sequencing inarguably produces more data, including the noncoding bases and structural and haplotype phasing information that exomes miss. But what most of those nucleotides do remains a mystery, as are the functional consequences of changing them. The implications of a missense mutation in a protein-coding gene, though, are more easily grasped. “The exome right now is the part of the genome we know how to interpret,” says Stacey Gabriel, director of the genomics platform at the **Broad Institute of Massachusetts Institute**

of Technology (MIT) and Harvard University.

As a result, researchers have been sequencing exomes at a blistering pace. Only a handful of papers on exome sequencing had been published by early 2010; today, there are more than 1,600 listed in PubMed.

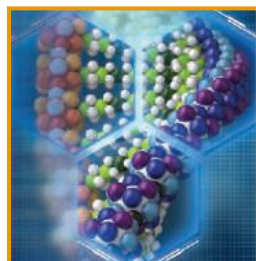
“In excess of one-hundred-some thousand exomes” have been decoded at the Broad Institute, says Gabriel. (Compared with the approximated 17,000 whole human genomes that have been sequenced to date, by Harvard University geneticist George Church's estimate.) Gabriel says her facility probably sequences four exomes for every whole genome and has a capacity of some 2,000 exomes per week. That's thanks to a fleet of 50 or so **Illumina HiSeq 2000s** and 2500s that were installed largely for the National Heart, Lung, and Blood Institute's Grand Opportunity Exome Sequencing Project, a gene-discovery project for which the Broad Institute and the **University of Washington** collectively sequenced 7,500 exomes.

Yale University's 10 HiSeqs decoded 12,000 exomes just in the past year, says **Howard Hughes Medical Institute** Investigator Richard Lifton, chair of the university's Department of Genetics, who published one of the first examples of exome sequencing in the clinic in 2009 and 15 additional exome papers since. “We're in a very productive phase right now,” Lifton says. “We're now sequencing exomes for an all-in cost, including all of the instrument amortization, of \$500 flat.” By comparison, he says, his lab has done “very few” whole genomes.

From the sequencer's point of view, though, it's six-of-one—both exomes and whole genomes are decoded the same way. The difference lies upstream, during the target capture and library preparation steps that precede sequencing.

Upcoming Features

Cell Culture: Scaling Up—December 6
RNA Technologies—January 17
Proteomics—February 21





HYBRIDIZATION STRATEGIES

Exome sequencing is simply a special form of target enrichment, a sequence preparation strategy that pulls out genetic elements of interest prior to decoding them. Researchers do this to stretch dollars and maximize efficiency: If you don't need the entire genome, why sequence it? At the same time, by sequencing fewer bases per sample, researchers can sequence more of those samples at once, and at higher coverage.

Coverage describes the number of times a given base is read by the sequencer. But such figures are statistics, not absolutes. Thirty-fold coverage means that each base is read 30 times on average; some are read more frequently, others less so. For many applications that's sufficient. But when it comes to finding rare variants on which clinical decisions might depend—a key mutation in a heterogeneous tumor, for example—the more passes at a given nucleotide, the better.

Some researchers use target enrichment to select a handful or a few hundred genes for sequencing. In exome sequencing, the target is the entire exon complement of the human genome. Typically, that's about 30 Mb of sequence, though users can supplement that pool with 5' and 3' untranslated regions, microRNAs, long noncoding transcripts, and other selected custom regions, which can significantly expand the amount of captured material. Illumina's Nextera Rapid Capture Exome kits, for instance, come in two flavors: a basic kit that captures 214,405 exons totaling 37 Mb and an "expanded" kit that adds UTRs and microRNAs for a total of 62 Mb.

Several of the earliest exome studies, including seminal 2009 reports by Lifton and Jay Shendure, associate professor of genome sciences at the University of Washington, employed array-based hybridization for target capture, a strategy then commercialized by **Agilent Technologies** and **Roche NimbleGen**. But solution hybridization (which uses a pool of biotinylated oligonucleotides that are pulled down post-hybridization with streptavidin beads) has now supplanted that approach.

Indeed, NimbleGen has since exited the microarray business altogether; Agilent still sells arrays, but mostly for customers who want to continue using the same enrichment tools they used earlier in a project, says Yong Yi, Agilent's marketing director for next generation sequencing products. "The vast majority of exomes generated today have been through solution hybridization," says Shendure.

Shendure was one of the first researchers to tap exomes for analyzing Mendelian disorders, and since 2009, by his count, "at least 100 new disease genes have been identified" using the technology. "It's kind of exploded pretty remarkably," he says, adding that there's nothing magic about exomes over whole genomes; they simply provide "a cost-accessible way of getting at most of what you wanted [from the genome] for a lot of the questions that are reasonable to ask."

In their exome work, Shendure and his colleagues use NimbleGen's solution-based SeqCap EZ Human Exome Library v3.0. So, too, does Lifton, whose lab has used the approach to identify genes that contribute to hypertension, congenital heart disease, autism, and thrombosis, among other conditions.

Illumina's Nextera Rapid Capture Exome kit (a high-speed method that uses transposons and optimized hybridization steps to simplify and shorten the protocol from several days to just a day and a half) is also based on solution hybridization, as is Agilent's SureSelect, a tool developed (and still used) at the Broad Institute that captures target sequences in solution using 120-mer biotinylated RNA baits—882,000 of them in the case of the SureSelect Human All Exon V5+UTRs panel.



MOLECULAR PADLOCKS

"When you're interested in sequencing a modest number of genes in a very large cohort of people, the padlock approach is a great one."

Targeted capture can also be accomplished using standard PCR (for instance, using **RainDance Technologies'** droplet-based approach). Or, users can try so-called molecular inversion probes (MIPs), or "padlock" probes.

A padlock probe, Church explains, is "basically two PCR primers that are joined at the hip." Linked in a single oligonucleotide, these two primers capture either end of the targeted sequence, forming a molecular semicircle (like an open padlock) that flanks a gap. The lock is closed using DNA polymerase and ligase, and the captured material amplified and sequenced, while nontargeted sequences are destroyed by exonucleases.

The approach offers a significant advantage over plain PCR: ease of multiplexing. "You don't get into the N-squared problem that you have from putting together multiplex PCR," Church explains, where "every primer can in principle interact with every other primer and all their extension products."

As a result, vast probe panels can be combined in a single reaction. In 2007, Church and his then-postdoc Shendure multiplexed 55,000 MIPs capable of capturing some 10,000 exons; in 2009, Shendure refined the pool to target 50,000 exons.

Today, Shendure favors SeqCap EZ. But he uses MIPs, too. In December 2012, he and his colleague Evan Eichler used them to capture 44 genes potentially related to autism spectrum disorders from 2,446 patient samples. Boston-based startup Pathogenica (co-founded by Church) also uses MIPs for bacterial strain typing and viral drug resistance analyses.

"When you're interested in sequencing a modest number of genes in a very large cohort of people, the padlock approach is a great one," Shendure says.

Agilent commercializes a somewhat related strategy in its HaloPlex Exome kit. In HaloPlex, long biotinylated oligos with capture sequences at either end capture targeted genomic fragments by hybridization, producing a circle that can be PCR amplified to generate a sequencing library in essentially a single step. "It's a combination of both worlds," Yi says. "It combines the advantages of hybridization with the simplicity of PCR."

EXOMES IN THE CLINIC

The power of exome sequencing was beautifully illustrated in a 2011 Pulitzer Prize-winning feature in the *Milwaukee Journal Sentinel*, which detailed the efforts of a group of researchers at the Medical College **continued**

Featured Participants

Agilent Technologies
www.agilent.com

Broad Institute
www.broadinstitute.org

Harvard University
genetics.med.harvard.edu

Illumina
www.illumina.com

Life Technologies
www.lifetechnologies.com

Pathogenica
www.pathogenica.com

Radboud University Medical Center
www.ru.nl/english

RainDance Technologies
raindancetech.com

Roche NimbleGen
www.nimblegen.com

University of Washington Genome Sciences
www.gs.washington.edu

Wellcome Trust Sanger Institute
www.sanger.ac.uk

Yale University
medicine.yale.edu/genetics

Additional Resources

International Cancer Genome Consortium
www.icgc.org

Milwaukee Journal Sentinel story
www.jsonline.com/features/health/111224104.html

NHLBI Grand Opportunity Exome Sequencing Project
esp.gs.washington.edu/drupal

The Cancer Genome Atlas
cancergenome.nih.gov

of Wisconsin to diagnose and treat a young boy with an inexplicable and exceptionally severe case of inflammatory bowel disease. In what turned out to be the first clinical use of exome sequencing, researchers identified a single point mutation in the X-linked inhibitor of apoptosis (XIAP) gene. That information suggested a possible therapeutic strategy, umbilical cord blood transplant. Since then, the college has applied the approach to 25 additional cases, obtaining a “definitive diagnosis” in 27% of them.

The Wisconsin researchers read their XIAP patient’s exome to 34x. Veltman says he would prefer to sequence his clinical exomes to 1,000x, but cost and throughput limit him to typically 60-fold coverage, while most of the exomes Broad’s Gabriel collects for her work on The Cancer Genome Atlas (TCGA) project are at 120-fold coverage. That’s far deeper than the typical whole genome—the Broad Institute sequences those to 50x, Gabriel says—but for some applications, and especially if patients are involved, even 120-fold coverage won’t do.

Pancreatic tumor samples, for instance, are notoriously difficult to obtain at high purity, Gabriel says, and require 300- to 400-fold coverage. For other applications, for instance, when looking for specific deleterious mutations in patient samples, physicians “want to have 500x coverage minimum,” Gabriel says.

That high depth of coverage, combined with the fact that—even in the exome—relatively few gene mutations are actually actionable, has some researchers contemplating a scaled-down approach in the clinic.

“The reality is for every clinical exome, one would be able to sequence many, many more [samples] in the setting of a targeted gene screen,” says Ultan McDermott, a principal investigator in the Cancer Genome Project at the **Wellcome Trust Sanger Institute**, adding, “It’s almost certain that defining the mutational signatures that underpin biological outcomes such

as drug response and survival will require an order-of-scale larger numbers than all of the previous international [next generation sequencing] efforts.”

As a result, McDermott advocates using large-scale exome-sequencing projects like the International Cancer Genome Consortium to identify interesting variants and smaller-scale targeted gene sequencing of four or five hundred genes to actually test for in patients. Indeed, McDermott says that approach already is being implemented in a pilot study called SPECTAcOLOR now kicking off in Europe, wherein all colorectal cancer patients being considered for inclusion in clinical trials will have 400 or so genes sequenced at the Sanger Institute. “This information would instantaneously allow the patient to be stratified into any clinical trial that arises where you need to know a particular mutational event as a point of entry.”

The flip side, of course, is that targeted studies are inherently biased. “You’re obviously not going to find anything that you haven’t specifically targeted,” McDermott says.

Veltman’s research is a case-in-point. Veltman studies severe intellectual disability. Perhaps 200 genes have been linked to that condition, he says, but “it’s clear that we know perhaps only 10%” of them. As a result, a targeted approach that looks only at known genetic players might well miss something interesting.

In one 2010 study, Veltman’s team sequenced 10 “trios”—an affected child and his or her unaffected parents—to 42-fold coverage, ultimately focusing on 10 “de novo” mutations (lesions not present in the parents). Three of these were known to be associated with intellectual disability, and three appeared irrelevant. Functional analysis of the remaining four—their observed behavior and interaction partners in model organisms, for instance—suggested they too could play a role in intellectual disability. A follow-up study in 2012 applied the same strategy to 100 patients, identifying de novo mutations in 10 genes known to be associated with intellectual disability and 19 candidate genes, and confirmed three additional novel genetic players in the condition (one of which was among the four picked up in the 2010 study).

The prevalence of de novo mutations in these two studies, Veltman says, flies in the face of the classical view of sporadic severe intellectual disability, which typically attributes the condition to autosomal recessive inheritance, and suggests a fundamental reassessment of the way geneticists think about rare diseases could be in order. “It turns out that these de novo mutations are a very common cause of intellectual disability,” Veltman says.

And yet many of them would have been missed using a simple gene panel as they were not among the many genes then known to be associated with severe intellectual disability.

Of course, even more can be discerned from whole genome analyses, and Veltman (like many others) is pursuing these, too. Most researchers agree that once the cost and ease of sequencing and interpreting whole genomes matches exomes, the attraction of exome sequencing will fade.

But it won’t disappear—if nothing else, large patient population studies may well require it. Says Shendure, “You should rationally do the study that makes the most sense for the question you’re interested in. Sometimes it will be genomes, sometimes it’ll be exomes. Sometimes it’ll be much more targeted.”

Jeffrey M. Perkel is a freelance science writer based in Pocatello, Idaho.

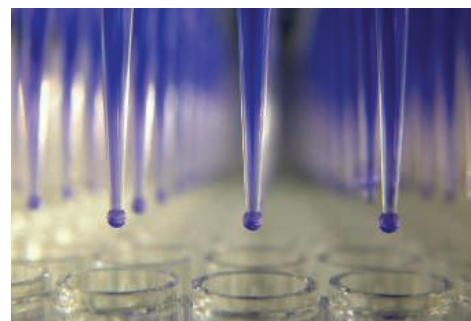
DOI: 10.1126/science.opms.p1300079

DNA PURIFICATION

MagSi-DNA cleanFIX offers a cost-effective solution for purifying DNA fragments, removing all unwanted side products and reagents. MagSi-DNA cleanFIX enables a combination of polymerase chain reaction (PCR) cleanup and dye terminator removal from sequence reaction mixes with a single product. The kit uses magnetic silica bead technology and is easily automated because no columns or centrifugation steps are involved. The kit uses a simple “Bind, Wash, and Elute” procedure common to magnetic particle purification protocols. The precision and reproducibility of the workstation in combination with the customized magnets for MagSi-DNA cleanFIX allow fast magnetic separation and homogenization of the samples. Overall, the PCR cleanup protocol using MagSi-DNA cleanFIX is shown to enable fast and efficient recovery of DNA fragments larger than 80 bp with >99% removal of primers and primer dimers. In addition, the dye terminator removal method consistently delivered high-quality sequence data with Phred >20 scores above 700.

AMS Biotechnology

For info: +44-(0)-1235-828200 | www.amsbio.com



DNA ENRICHMENT KITS

PointMan DNA Enrichment kits comprise three kits for enriching mutations in BRAF, KRAS, and EGFR T790M genes associated with skin melanoma, colorectal, and lung cancers. PointMan kits offer highly specific and ultrasensitive enrichment of mutant genes in a background of wild-type (normal) genes that is unmatched by existing technologies. PointMan is a real-time polymerase chain reaction (PCR) technology that provides reliable and extremely sensitive detection for cancer mutations. It is highly efficient in amplifying the target sequence of interest, while suppressing amplification of the wild type. The resulting sample is effectively enriched for the mutation, thereby having the potential to offer industry leading sensitivity in a wide variety of sample types. PointMan DNA enrichment kits can also be used to enrich all mutant sequences within the gene of interest using a single set of reagents, unlike competing technology that requires a separate reagent set for each mutation within a gene sequence.

EKF Diagnostics

For info: +44-(0)-2920-710570 | www.ekfdiagnostics.com

DNA METHYLATION ANALYSIS

TrueMethyl brings unprecedented clarity to the analysis of DNA by providing quantitative, accurate, and repeatable single-base resolution sequencing of the modified bases hydroxymethylcytosine (5-hmC) and methylcytosine (5-mC) for the first time. Traditional bisulfite sequencing cannot discriminate between 5-hmC and 5-mC. Recent studies have shown that at some sites in the genome the level of 5-hmC can be comparable to the level of 5-mC, emphasizing the importance of identifying these variants accurately. The high-quality and easy-to-use TrueMethyl kits utilize innovative oxidative bisulfite sequencing (oxBS-Seq). TrueMethyl kits can be used with a variety of common platforms including next generation sequencing systems, methylation arrays, and targeted assays. Trials of the kits conducted at leading research centers around the world have already begun to yield new insights into genome function and highlighted the advantages of TrueMethyl relative to traditional approaches.

Cambridge Epigenetix

For info: 800-754-5916 | www.cambridge-epigenetix.com

DIGITAL PCR ASSAYS

PrimePCR assays, for Droplet Digital polymerase chain reaction (ddPCR), are predesigned assays for mutation detection and copy number variation (CNV), which have been experimentally validated to provide single-copy PCR resolution without a standard curve. Droplet Digital PCR technology provides an absolute measure of target DNA molecules. The assays, combined with the sensitivity of Bio-Rad's ddPCR system, are capable of detecting a single mutant copy in a background of 2,000 or more wild-type molecules (0.05% mutation frequency). The precision of ddPCR also enables cancer researchers to discriminate small-fold copy number changes with the 62 assays targeting common cancer genes and two reference target assays now available. The PrimePCR ddPCR assays employ universal cycling conditions and do not require optimization. Primer specificity has been validated by next generation sequencing, and all assays have been validated on Bio-Rad's QX100 Droplet Digital PCR system. PrimePCR ddPCR assays are available in multiple reaction sizes.

Bio-Rad

For info: 800-424-6723 | www.bio-rad.com/PrimePCR

GENOME EDITING TOOLS

Sigma CRISPRs is an inexpensive mammalian genome editing tool suitable for screening and exploratory studies. Sigma CRISPRs are packaged in a single plasmid vector containing GFP for fast enrichment of gene-edited cells and can be custom-designed online using an exclusive bioinformatics tool. Derived from bacterial and archaeal immune defenses, which guard against invading viruses and foreign DNA, Sigma CRISPRs utilize customizable RNA—nuclease complexes that allow introduction of genome edits at a desired site in mammalian genomes with high efficiency. Sigma's exclusive online design tool allows users to identify the best target site(s) within human, mouse, and rat protein-coding exons that adhere to CRISPR/Cas targeting requirements. It is the only commercial tool that minimizes the possibility of off-target effects by automatically applying CRISPR/Cas best design practices, including a minimum requirement of three base-pair mismatches between the target site and other sites in the genome.

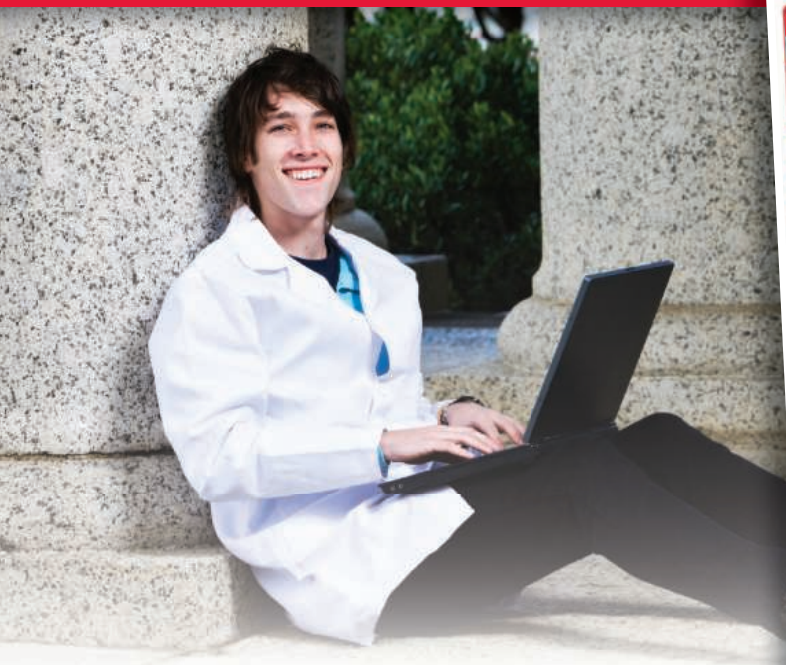
Sigma-Aldrich

For info: 800-325-3010 | www.sigma.com/crisprs

Electronically submit your new product description or product literature information! Go to www.sciencemag.org/products/newproducts.dtl for more information. Newly offered instrumentation, apparatus, and laboratory materials of interest to researchers in all disciplines in academic, industrial, and governmental organizations are featured in this space. Emphasis is given to purpose, chief characteristics, and availability of products and materials. Endorsement by *Science* or AAAS of any products or materials mentioned is not implied. Additional information may be obtained from the manufacturer or supplier.

For your career in science, there's only one **Science**

A career plan customized
for you, by you.



myIDP.sciencecareers.org



Recommended by leading professional societies and endorsed by the National Institutes of Health, an individual development plan will help you prepare for a successful and satisfying scientific career.



In collaboration with FASEB, UCSF, and the Medical College of Wisconsin and with support from the Burroughs Wellcome Fund, AAAS and *Science* Careers present the first and only online app that helps scientists prepare their very own individual development plan.

Visit the website and
start planning today!
myIDP.sciencecareers.org

In partnership with:



Luminex® Multiplex Immunoassays from R&D Systems

Analyze cancer serum samples:

Inflammation markers	Angiogenesis factors	Matrix remodeling factors
Angiopoietin-2	Angiogenin	EMMPRIN
IL-1 α	Angiopoietin-1	MMP-1
IL-1 β	Endostatin	MMP-2
Chitinase 3-like 1	FGF acidic	MMP-3
Cripto-1	FGF basic	MMP-7
DcR3	PDGF-AA	MMP-9
IL-12 p70	PDGF-BB	TIMP-1
IL-17A	PIGF	TIMP-2
IL-18 BP α	+ VEGF	TIMP-3
+ TNF- α	Luminex Assay	+ TIMP-4
Luminex Assay		Luminex Assay

Exclusively available from R&D Systems

One simple solution for profiling your complex samples

The world leader in the development of single analyte immunoassays now offers the widest selection of Luminex bead-based multiplex kits. Several features of our Luminex products distinguish us from the competition:

- We uniquely offer two different types of Luminex Kits: Luminex Screening & Luminex Performance Assays.
- We have the widest selection of analytes with over 20% of our menu being exclusively available from R&D Systems -160 screening analytes, 16 performance panels.
- We have developed the simplest online ordering tool for researchers to design their own user-defined kits.

Find your multiplex solutions

| RnDSystems.com/Luminex



Luminex is a registered trademark of Luminex Corporation.

There's only one

Science



Science Careers Advertising

For full advertising details, go to ScienceCareers.org and click For Employers, or call one of our representatives.

Tracy Holmes

Worldwide Associate Director
Science Careers
Phone: +44 (0) 1223 326525

THE AMERICAS

E-mail: advertise@sciencecareers.org
Fax: 202-289-6742

Tina Burks

East Coast/West Coast/South America
Phone: 202-326-6577

Marci Gallun

Midwest/Canada
Phone: 202-326-6582

Candice Nulsen

Corporate
Phone: 202-256-1528

Online Job Posting Questions

Phone: 202-312-6375

EUROPE / INDIA / AUSTRALIA / NEW ZEALAND / REST OF WORLD

E-mail: ads@science-int.co.uk
Fax: +44 (0) 1223 326532

Axel Gesatzki

Phone: +44 (0) 1223 326529

Sarah Lelarge

Phone: +44 (0) 1223 326527

Kelly Grace

Phone: +44 (0) 1223 326528

JAPAN

Yuri Kobayashi

Phone: +81-(0)90-9110-1719
E-mail: ykobayas@aaas.org

CHINA / KOREA / SINGAPORE / TAIWAN / THAILAND

Ruolei Wu

Phone: +86-1367-1015-294
E-mail: rwu@aaas.org

All ads submitted for publication must comply with applicable U.S. and non-U.S. laws. Science reserves the right to refuse any advertisement at its sole discretion for any reason, including without limitation for offensive language or inappropriate content, and all advertising is subject to publisher approval. Science encourages our readers to alert us to any ads that they feel may be discriminatory or offensive.

Science Careers

From the journal Science



ScienceCareers.org



Senior Faculty Position

The Center for Immunology & Microbial Disease at Albany Medical College invites applications for a tenure-track Associate/Full Professor position from individuals with demonstrated research productivity and interest in translational research. Applicants for this senior faculty position should have an MD, MD/PhD or PhD, and lead an internationally-recognized research program in immunology and/or host-pathogen interactions with particular emphasis on disease mechanisms in humans. Successful candidates will also demonstrate strong communication skills and an ability to excel in collaborative research endeavors. The position will have a critical role in the development of joint research programs between the Interdisciplinary Research Center in Immunology and Microbial Disease and the Departments of Internal Medicine and Pediatrics. This position will also have broad responsibility for leading a program for training of doctoral and physician research scientists emphasizing host-pathogen interactions. Joint appointments in divisions of clinical interest are encouraged. The CIMD faculty comprises a highly collaborative research group that this year received \$4.6M in NIH funding, ranking it within the top half of all Microbiology and Immunology programs in the country. The successful candidate will receive an attractive start-up package including the ability to recruit and lead a focused group to be located in our newly constructed 4500 sq ft dedicated laboratory space. This new facility has access to all departmental core services including the Center's fully-staffed Immunology and ABSL-3/BSL-3 Cores. We also have a cooperative program with Translational Medicine that includes use of a new Clinical Research Unit capable of performing both investigator-initiated and industry-sponsored Phase 1/2 infectious disease clinical trials. Albany Medical College is located in a mid-sized city within the upstate New York Capital Region, and has easy access to Boston, New York City, and the Adirondack Mountains.

Applicants should send their curriculum vitae, a statement of research plans, and contact information for three references to:

Faculty Search Committee
Center for Immunology & Microbial Disease and
Infectious Diseases Program, Department of Internal Medicine
Albany Medical College
47 New Scotland Avenue, MC-151
Albany, NY 12208

For further information about the Center, visit www.amc.edu/Research/IMD/

AMC supports a diversified, smoke-free environment and is proud to be an Equal Opportunity/Affirmative Action Employer, encouraging women and minorities to apply. In support of a safe, drug-free environment, criminal background checks and drug testing are part of our hiring process.



LSUHealth
NewOrleans

Professor and Department Head
Department of Genetics
Louisiana State University Health Sciences Center
School of Medicine

The Louisiana State University Health Sciences Center School of Medicine in New Orleans invites applications and nominations for Head of the Department of Genetics. The successful candidate will have a PhD, MD or MD/PhD, will be nationally recognized for research in Genetics or Genomics, and will have an active research program funded through competitive research grant programs; the successful candidate will satisfy requirements for appointment at the professorial rank.

The successful candidate will demonstrate a commitment to biomedical research and education and will have documented leadership ability and the capacity to provide a vision of excellence for the Department. Achievements in multi-disciplinary, collaborative research, mentorship, teaching, and administration are essential; candidates must be committed to promoting an inclusive environment in the Department. The incumbent will be responsible for all facets of activity in the department, including both graduate and undergraduate medical education, faculty recruitment and retention, and development of research programs. He/she will be expected to coordinate the development of departmental and collaborative research programs within the school and with our academic partners. We are especially interested in candidates who can forge strong collaborative research programs, such as program/project and center grants and funded training grants, and candidates who will foster meaningful translational research programs.

Opportunities for professional growth and leadership reside in conjunction with other components of the LSUHSC community, including the School of Public Health, the Children's Research Institute, the Neuroscience, Cancer and Cardiovascular Centers, the NIH-designated Alcohol Research Center, and the Louisiana Vaccine Center. Excellent core laboratory facilities are available, including proteomics, genomics, imaging, and bioinformatics. Outstanding opportunities exist for the growth of programs in research and education in clinical genetics. The construction of new academic university and Veterans Administration medical centers adjacent to the LSUHSC campus, due to open in 2015 and 2016, will provide state-of-the-art facilities supporting clinical programs for our traditional and other patient constituencies. Resources will be available for departmental development, and for faculty recruitment, including an endowed chair for genetic research in hearing loss, with the likelihood of further endowed chairs focused on specific disease entities. The compensation package is highly competitive and the University offers a strong benefits program.

Women and under-represented minority candidates are especially encouraged to apply. Candidates should provide their Curriculum Vitae, including a full list of publications, a brief statement of educational, research, service, and administrative interests, and the names and contact information of three references. These materials should be forwarded electronically to: **Karen Eigenbrod (keigen@lsuhsc.edu)** Reference **#82 41016 026**. Review of applications will commence immediately and will continue until the position is filled.

LSUHSC is an Equal Opportunity/Affirmative Action Employer.

ENGINEERING AT ILLINOIS

DRIVE YOUR VISION

ENDOWED CHAIRS AND PROFESSORSHIPS IN BIG DATA

Big Data will be the engine for the next generation of discoveries. As one of the Top 5 programs in the world, Engineering at Illinois has a head start and we plan to keep it. Thanks to the \$100-million Grainger Engineering Breakthroughs Initiative, we're creating more than 35 new endowed professorships and chairs in Big Data and other fields. Applications and nominations are being accepted now. If you're ready to drive the future of Big Data, Illinois is the place for you.

GraingerInitiative.engineering.illinois.edu



Illinois is an Affirmative Action/Equal Opportunity Employer. www.inclusiveillinois.illinois.edu. Full consideration will be given to applications and nominations received by December 16, 2013.



College of
Biological Sciences
UNIVERSITY OF MINNESOTA
Driven to DiscoverSM

Are you a creative investigator who excels at working collaboratively and pushing disciplinary boundaries?

Then we want to hear from you.

The College of Biological Sciences at the University of Minnesota is hiring faculty for **three interdisciplinary research clusters in emerging areas of biology: genome variation, cellular biophysics and synthetic biology.**

All positions are tenure track. Successful candidates will have research expertise that complements current faculty and be committed to graduate and undergraduate education.

Learn more at
z.umn.edu/cbsclusterhiring

The University of Minnesota is an equal opportunity educator and employer.

UNIVERSITY of
HOUSTON

COLLEGE OF PHARMACY
**Faculty Position in Immunology
Department of Pharmacological and
Pharmaceutical Sciences**

The Department of Pharmacological and Pharmaceutical Sciences at the University of Houston is accepting applications for a tenure-track faculty position in Immunology at either the Associate Professor or Professor level. The University of Houston is Carnegie-designated Tier1 public research university and also is a member institution of the world-renowned Texas Medical Center which houses major academic research programs in cancer, genetics, and neuroscience with a major focus on translational research. The successful candidate will be provided lab space will receive a start-up package commensurate with their qualifications. Department faculty members teach in the Pharm.D. professional program and also participate in departmental Ph.D. programs in Pharmacology and Pharmaceutics. The search is focused on adding expertise in the immunology, immunotherapy and vaccines along with other areas relevant to drug development.

Eligible candidates must have an earned doctoral degree, postdoctoral experience and a strong track record for establishing and maintaining an excellent extramurally-funded research program. Applications will be accepted until the position is filled, although an early application is recommended. Interested individuals should send a letter describing his/her research program, a curriculum vitae and the names of three references with postal and Email addresses, telephone and FAX numbers electronically in PDF format to:

Tahir Hussain, Ph.D. , Chair, Search Committee
c/o ppsimmun@uh.edu
University of Houston
College of Pharmacy
Houston, TX, 77204-5037

Web site: <http://www.uh.edu/pps/pps/index.html>

The University of Houston is an Affirmative Action/Equal Opportunity Employer. Minorities, women, veterans, and person with disabilities are encouraged to apply.



Washington University in St. Louis

SCHOOL OF MEDICINE

**Faculty Positions in Biochemistry and
Molecular Biophysics**

The Department of Biochemistry and Molecular Biophysics at Washington University School of Medicine invites applications for tenured and tenure-track faculty positions in computational biophysics and biochemistry.

Outstanding individuals working in the development and applications of computational approaches to the study of biological molecules are encouraged to apply. Research programs of successful candidates will have a focus on the next generation of computational methodology to describe fundamental principles of molecular mechanisms in basic science and medicine.

Research in the department spans a wide range of topics including membrane proteins, molecular motors, nucleic acid/protein interactions, molecular structure and dynamics, and signal transduction. The university environment is rich with opportunities for collaboration in a wide range of disciplines. Additional information about the department is available at <http://www.biochem.wustl.edu>.

Applicants should email their curriculum vitae and a brief description of their research interests to the Search Committee at search@biochem.wustl.edu. Applicants should include contact information for three individuals who can write letters of recommendation. The committee will request letters as necessary. **Completed applications will be reviewed on a rolling basis, starting immediately. For full consideration, applications should be received by January 1, 2014.**

Washington University is an Equal Opportunity Employer. We are committed to the recruitment of candidates traditionally underrepresented on university faculties. Individuals of any race, ethnicity, gender or sexual orientation are encouraged to apply, as are disabled individuals and veterans. The School of Medicine at Washington University is committed to finding solutions to global health problems, including ones that affect minority and disadvantaged populations.



University of Minnesota
Medical School

**INFECTIOUS DISEASES – VIROLOGY
TENURE TRACK**

The Program in HIV Medicine at the University of Minnesota is recruiting for a full-time faculty member at the Assistant, Associate, or Full Professor level in the physician/scientist tenure track pathway to join the newly established Program in HIV Medicine. The mission of this program is to investigate innovative strategies that will contribute to curing HIV infection and fully restore immunity. The successful candidate will have a MD, PhD, or MD/PhD degree and will focus on creating an interdisciplinary, extramurally funded research program to identify mechanisms of viral persistence in tissue reservoirs of HIV infection. This individual will have the opportunity to collaborate with faculty in the Center for Immunology, Department of Microbiology, Center for Drug Design, Center for Infectious Disease and Microbiology Translational Research, and the Institute for Molecular Virology at the University of Minnesota. The successful candidate will also have the opportunity to provide outpatient care for HIV-infected patients at the University of Minnesota HIV/Infectious Diseases clinic (if a licensed MD in the State of Minnesota) and to do inpatient ID consulting. Salary will be commensurate with qualifications and expertise.

Applicants should send a letter of interest describing their research interests and expertise, along with current curriculum vitae and the names of three references, by e-mail to: **Timothy Schacker, MD, Director of the Program in HIV Medicine, c/o: Lisa Turnquist (turnq007@umn.edu).**

The University of Minnesota School of Medicine is an Equal Opportunity, Equal Access, Affirmative Action Employer.



Talents Recruitment

Institute of Agro-Products Processing Science and Technology, Chinese Academy of Agricultural Sciences Beijing, China

Authorized by the State Council, Ministry of Agriculture, and Ministry of Science and Technology, the Institute of Agro-Products Processing Science and Technology (IAPPST), Chinese Academy of Agricultural Sciences (CAAS) was founded on the base of the Institute of Atomic Utilization in Agriculture in October, 2002. As the only national public welfare research institute on agro-products processing, the Institute carries on the responsibility of basic and application fundamental research, high-tech development, key technology study and products development; solving the fundamental, directional, national and strategic scientific issues in China's agro-products processing. The Institute gives full play to leadership as the only national research institute on agro-products processing, to devote on scientific research and technology innovation, technologies' transfer and application in industry, international cooperation, senior talents cultivation and industrial policy research, etc., to build the first class research institution in China and the world.

With the aim of building the world class research institute in the world, based on the requirements of Scientific Innovation Project of CAAS, authorized by CAAS, IAPPST now recruits scientists from China and abroad.

I Job Positions

1. Cereal and Oil Pressing and Comprehensive Utilization

(1) Grain and Oil Processing

Applicant Eligibility

- Professional background on processing suitability evaluation of staple grain and oil plants; production and modification of plant protein; comprehensive utilization of grain by-products and functional factors extraction; preparation of functional oligopeptide, polysaccharide, and related biological activity mechanism;
- An overseas doctor degree or overseas working experience (minimum of three years) after PhD; Under 40 years old;
- Presided at least one Project of National Natural Science Foundation of China or National Research Project;
- Published at least one paper included by SCI with IF>5 as first author or corresponding author or has Provincial Prize (ranked in top 3)

(2) Potatoes Processing

Applicant Eligibility

- Professional background on potato processing and comprehensive utilization; extraction and production of nutrient and functional factor from potato; nutritional and functional evaluation of potato;
- An overseas doctor degree or overseas working experience (minimum of three years) after PhD; Under 40 years old;
- Published at least 3 papers as first author or corresponding author in core journal or at least one paper with IF > 5.

2. Meat Processing and Quality

Applicant Eligibility

- Professional background on meat quality and safety, meat engineering and technology, comprehensive utilization of animal bone and blood technology;
- An overseas doctor degree or overseas working experience (minimum of three years) after PhD; Under 40 years old;
- Published at least 3 papers in core journal as first author or corresponding author, or at least have one paper with IF > 5; or has Provincial Prize (ranked in top2).

3. Traditional Food Processing and Machinery

Applicant Eligibility

- Professional background on engineering and processing technologies for the industrialization of traditional Chinese food, traditional Chinese food processing equipment design, manufacture and automation;
- An overseas doctor degree or overseas working experience (minimum of three years) after PhD; Under 45 years old;
- Presided independently national or provincial research projects; published papers in core journal included by SCI with IF >3 as first author or corresponding author; or has provincial (or above) prize (rank in top 3) or get international patent for invention;
- Priority for people with National Distinguished Youth Science Foundation.

4. Fruit and Vegetable Processing and Comprehensive Utilization

Applicant Eligibility

- Professional background on fruit and vegetable functional components analysis and evaluation, varying pattern, processing control theory and technology;

- Doctor degree; Under 40 years old; at least 3 years continuous overseas research experience;
- Published at least 1 papers included by SCI with IF >5 as first author or corresponding author, or the total IF of papers >10;
- Presided 2-3 research projects in abroad as core scientist.

5. Preservation and Logistics

Applicant Eligibility

- Professional background on cold chain logistics technology and equipment, new product research and development on cold chain logistics; preservation equipment and preservatives; molecular mechanism of quality control during storage of agro-products;
- Doctor degree; Under 45 years old; at least 3 years continuous overseas research experience;
- Published at least 2 papers in core journal as first author or corresponding author, or at least have one paper with IF > 5;
- Has important patent for invention;
- Priority for people with National Distinguished Youth Science Foundation.

6. Bio-active Factors and Functional Food

Applicant Eligibility

- Professional background on bio-active factors; functional evaluation and mechanism research on bio-active factors; development of functional food;
- Doctor degree; Under 45 years old; at least 3 years continuous overseas research experience;
- Published at least 5 papers in core journal as first author or corresponding author, or at least one paper with IF >10; or 1-2 significant patent for invention;
- Priority for People with National Distinguished Youth Science Foundation.

7. Nutrition and Health

Applicant Eligibility

- Professional background on food nutrition; structure-function relation of nutrients and nutrition therapy, personalized nutrition design and healthy food;
- Doctor degree; Under 45 years old; at least 3 years research experience on food nutrition and health;
- Published at least 5 papers in core journal as first author or corresponding author, or at least one paper with IF >10.

8. Bio-processing and Food Biotechnology

Applicant Eligibility

- Professional background on directional enzymatic hydrolysis and transformation of biomass, targeting technology of protein or polysaccharide, novel biomaterials and novel biological food;
- Doctor degree; Under 45 years old; at least 3 years continuous overseas research experience;
- Published at least 5 papers in core journal as first author or corresponding author, or at least one paper with IF >10.

9. Fungus and Prevention

Applicant Eligibility

- Professional background on *Verticillium dahliae* and toxigenic fungi prevention and control technology;
- Doctor degree; Under 45 years old; at least 3 years continuous overseas research experience;
- Published at least 5 papers in core journal as first author or corresponding author, or at least one paper with IF >10;
- Priority for people with National Distinguished Youth Science Foundation, provincial or national prize owner.

10. Mycotoxin Prevention

Applicant Eligibility

- Professional background on formation and control mechanism, mycotoxin prevention and elimination theory and technology;
- Doctor degree; Under 40 years old; at least 3 years continuous overseas research experience;
- Published at least 5 papers in core journal as first author or corresponding author, or at least one paper with IF >10.

II Materials Required for Recruitment

Applicants should provide resume with list of publications, research interests, professional titles; PDF copies of certificates of academic achievements and documents for funded projects, patents, etc.

III Contact

Contact person: Ms. Meng Zhe

Telephone: 86-10-62815957 Email: zhbg5109@126.com

Fax: 86-10-62895382

Mail address: No. 2 Courtyard, Yuanmingyuan West Road, Haidian District, Beijing (5109 mailbox) Postcode: 100193

The Institute's website: <http://www.foodcaas.ac.cn/index.html>



**VANDERBILT
UNIVERSITY**

FACULTY POSITION IN MICROBIAL PATHOGENESIS
Department of Pathology, Microbiology and Immunology
Division of Host-Pathogen Interactions
Vanderbilt University School of Medicine

The Department of Pathology, Microbiology and Immunology at Vanderbilt University School of Medicine invites applications for a tenure-track faculty position at the Assistant Professor level (PhD, MD, MD/PhD). Areas of particular interest include, but are not limited to, viral pathogenesis, bacterial pathogenesis, and host-microbe interactions. Successful candidates will be expected to establish and maintain an independent research program and participate in teaching of graduate and medical students. Candidates should have substantial post-graduate training highlighted by peer-reviewed publications that demonstrate research productivity.

Vanderbilt University Medical Center, located on the Vanderbilt University campus, is home to internationally recognized programs in bioinformatics, drug discovery, global health, inflammation, imaging science, pharmacology, proteomics, and vaccine science. The School consistently ranks in the Top 20 US Medical Schools and provides outstanding opportunities for scholarship, collaboration, and teaching.

The Vanderbilt University campus is a National Arboretum located in the heart of Nashville, the capital of Tennessee. Known internationally as "Music City USA", Nashville is also the home to professional sports teams, the Nashville Symphony, the Frist Center for the Visual Arts, and numerous activities for outdoor enthusiasts. Nashville, Tennessee is a wonderful place to live, work, and raise a family.

Applicants should send a *curriculum vitae*, a statement of current and future research interests, and three letters of recommendation to: **Eric Skaar, Ph.D., Director, Division of Host-Pathogen Interactions, Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Room A-5102, Medical Center North, 1161 21st Ave. S., Nashville, TN 37232.** Inquiries, applications, and recommendation letters can be directed via email to **eric.skaar@vanderbilt.edu**.

*Vanderbilt University is an Affirmative Action/Equal Opportunity Employer.
 Women and minority candidates are encouraged to apply.*



COLUMBIA UNIVERSITY
 IN THE CITY OF NEW YORK

Neuroscience Faculty Recruitment

The Department of Neuroscience at Columbia University is currently recruiting faculty in the neurosciences. We have a particular interest in research programs that link neural circuitry and behavior in genetically-tractable mammalian model systems. We will also consider candidates addressing issues in cognitive neuroscience at the level of systems and circuits.

Columbia University has an exceptionally strong and broad program in the neurosciences and aims to enhance interactions between basic and clinical research and to link the neurosciences with a wide range of other disciplines within the University. New faculty will be affiliated with the Department of Neuroscience, with the Doctoral Program in Neurobiology and Behavior, and with the newly established Zuckerman Mind Brain Behavior Institute. In addition, there are numerous opportunities for interaction with other scientific departments and programs at the Medical Center and Morningside Heights campuses.

The application deadline is November 30, 2013. Please submit applications online at **<https://academicjobs.columbia.edu/applicants/Central?quickFind=58277>** and include a cover letter, curriculum vitae, and a statement of research interests. In addition, please arrange for three references to submit letters of recommendation.

Columbia University is an affirmative action/
 equal opportunity employer.

**THE
UNIVERSITY
OF RHODE ISLAND**

**GRADUATE SCHOOL
OF OCEANOGRAPHY**

THREE FACULTY POSITIONS IN OCEANOGRAPHY

The Graduate School of Oceanography at the University of Rhode Island invites applications for 3 tenure track faculty members. We seek applications for 1). Assistant Professor in marine biogeochemistry (posting: 6001261); 2). Assistant Professor in physical oceanography with an interest in submeso- and smaller-scale processes (posting: 6001252); and 3). Full Professor of Oceanography and Director of the Coastal Resources Center (posting: 6001259).

Located on the water's edge at URI's Narragansett Bay Campus, GSO is the state's center for marine studies, research and outreach and operates the R/V Endeavor. Students, faculty and staff collaboratively address the science questions and challenges of today. Each new faculty member will be expected to develop strong externally funded research programs, advise graduate students, and teach undergraduate and graduate courses.

Application review will begin January 7, 2014 and continue until the positions are filled. Visit <https://jobs.uri.edu> and search individual position numbers to read full position descriptions with required and preferred qualifications. Submit applications online, including the following in PDF format: (1) a letter of application; (2) curriculum vitae to include the names, email addresses, and telephone numbers of at least three references; and (3) a statement of teaching and research interests. Other relevant material in support of your application may be sent directly to the search chairs (Marine Biogeochemistry, Rebecca Robinson, rebecca_r@mail.uri.edu; Physical Oceanography, Arthur Spivack, spivack@mail.uri.edu; Professor/Director Coastal Resources Center, David Smith, dcsmith@mail.uri.edu). The University of Rhode Island is an AA/EEOD employer and values diversity.



UNIVERSITY AT ALBANY
 State University of New York

RNA Cancer Biologist Assistant Professor

The Department of Biological Sciences and The RNA Institute invite applications for a tenure track Assistant Professor position in cancer biology with a focus on the role of RNA in oncogenesis or cancer therapeutics, or an RNA based genomics approach to cancer biology.

The successful candidate will be a member of the Department of Biological Sciences and The RNA Institute (<http://www.albany.edu/rna>) which has state-of-the-art laboratories housed in the Life Sciences Research Building (<http://www.albany.edu/lifesciences>). The Institute brings together more than 35 investigators from the College of Arts & Sciences, the College of Nanoscale Science and Engineering, the School of Public Health, and regional institutions including the Wadsworth Center, Rensselaer Polytechnic Institute, and Albany Medical College. This creates an outstanding environment for research collaborations.

The successful candidate will be offered a competitive salary, start-up package, and research space.

Submit applications at:
<http://albany.interviewexchange.com/candapply.jsp?JOBID=42800>

Applications must include a CV with publications, present and past grant funding, statements of research interests and experience in RNA science and teaching interests, and a minimum of three letters of references as directed by the above website.

Qualifications: Ph.D. or M.D. from a college or university accredited by the U.S. Department of Education or an internationally recognized accrediting organization and a strong publication record reflecting significant scientific accomplishments. The applications must address the candidate's ability to work with and instruct a culturally diverse population. Preferred qualifications include productive post-doctoral training and the potential, or demonstrated ability, to obtain independent extramural funding. Review of applications will begin on November 15, 2013 and continue until the position is filled.

The University at Albany is an EEO/AA/IRCA/ADA employer.

GRADUATE SCHOOL OF

Quantitative Biosciences Munich



Do you wish to ...

- ... earn a PhD, building on your training in biochemistry, biology, physics, or applied maths?
- ... conduct cutting-edge research at the interface of experiment and quantitative theory?
- ... learn how to communicate and work with scientists from different fields?

Then join us at QBM!

The newly established Graduate School of Quantitative Biosciences Munich (QBM) is funded by the German Excellence Initiative and seeks to prepare young life scientists for the emerging era of quantitative, systems-oriented bioscience. It provides an innovative, international PhD training program that bridges the divide between traditionally separate disciplines, from biochemistry and medicine to bioinformatics, experimental and theoretical biophysics, and applied mathematics.

Key elements of the program are an interdisciplinary research project jointly supervised by two PIs from different fields, and an educational curriculum centered around an intensive core course that integrates a wide range of approaches to biological problems. A multi-faceted mentoring and professional skills program supports the students' growth as independent scientists.

For more information, visit us at www.qbm.lmu.de

Application deadline: **January 6, 2014**



MAX-PLANCK-GESELLSCHAFT

Call for Nominations



Max Planck Research Award 2014

The International Research Award of the Alexander von Humboldt Foundation and the Max Planck Society

The Alexander von Humboldt Foundation and the Max Planck Society jointly confer the Max Planck Research Award, which is funded by the German Federal Ministry for Education and Research, on exceptionally highly-qualified German and foreign scientists. The researchers are expected to have already achieved international recognition and to continue to produce outstanding academic results in international collaboration – not least with the assistance of this award.

Every year, two research awards are conferred on internationally renowned scientific researchers. One of the awards should be given to a researcher working in Germany and the other to a researcher working abroad. As a rule, each Max Planck Research Award is endowed with 750,000 Euros. Nominations of qualified female scientific researchers are especially welcome.

On an annually-alternating basis, the call for nominations addresses areas within the natural and engineering sciences, the life sciences, and the human and social sciences.

The Max Planck Research Award 2014 will be conferred in the area of natural and engineering sciences in the subject

Quantum Nano Science

113 years after the foundation of quantum theory by Max Planck, researchers succeed in controlling materials with ever higher precision to realize exotic quantum states. Thus nano structured materials and devices arise, that by exploiting the most bizarre features of quantum mechanics – take discretisation, superposition, entanglement and many body systems as examples - are designed for special purposes. Such phenomena form the focus of the relatively young experimental field of Quantum Nano Science that has emerged at the interfaces of nano science, quantum optics, photonics, materials technology and quantum information.

The Rectors/Presidents of German universities or research organisations and the scientific heads of institutes of these organisations are eligible to nominate candidates. Nominations must be submitted to the Alexander von Humboldt Foundation. Applications by prospective candidates themselves are not possible. The deadline for nominations is **31 January 2014**.

Further information can be obtained from the

Alexander von Humboldt-Stiftung, Bonn (Germany)
www.humboldt-foundation.de/web/max-planck-award.html
E-Mail: ursula.michels@avh.de

SPONSORED BY THE



Federal Ministry
of Education
and Research



Assistant Professor of Systems Biology Harvard Medical School

Applications are invited for an Assistant Professor position with a primary appointment in the Department of Systems Biology and an associate faculty appointment in the Center for Biomedical Informatics at Harvard Medical School.

The Department of Systems Biology studies the dynamic and quantitative behavior of systems of biological components — molecules, cells, organisms or entire species. The Center for Biomedical Informatics conducts informatics research with a strong emphasis on translational biomedicine informed by innovative computational strategies. Both groups have core interests in the application of computer science, statistics and mathematics to biomedical problems. This position is expected to help bridge the research areas of these two faculties.

The successful candidate will be a computational biologist using cutting-edge bioinformatics techniques that are rooted in a fundamental understanding of biophysical principles, and applied to problems of immediate medical relevance. Candidates with active research programs and demonstrated ability to provide insight into both basic biological problems and problems directly relevant to human health will be at a particular advantage.

The appointee will become a member of Harvard's Ph.D. Program in Systems Biology, a cross-Harvard interdisciplinary program that attracts extraordinary graduate students, as well as the Master of Medical Sciences in Biomedical Informatics (MMSc) degree program at CBMI. A doctoral degree is required.

The deadline for applications is **November 30, 2013**. Please submit a curriculum vitae, research proposal (≤4 pages), and PDFs of ≤3 publications. During the application process you will be asked to give the e-mail addresses of at least three colleagues who have agreed to write letters of recommendation for you.

Applications from, or nominations of, women and minority candidates are encouraged. Harvard is an Affirmative Action/Equal Opportunity Employer.



Plant Physiology Assistant Professor School of Biological Sciences College of Arts and Sciences

The School of Biological Sciences at Washington State University, Pullman, Washington, invites applications for a full-time, permanent, tenure-track faculty position in plant physiology. This position is to be filled at the Assistant Professor level and will begin in August of 2014. Candidates are expected to perform fundamental research that examines factors controlling or coordinating the physiology of plants, particularly mechanisms of resource acquisition and utilization. The ideal candidate will combine traditional physiological investigations and cutting edge approaches such as genomics, transcriptomics, metabolomics, dynamic imaging and microscopy, growth modeling and/or plant hyperspectral analysis. Areas of research may include but are not limited to photosynthesis, primary metabolism, and growth. The research should be relevant to current areas of interest in the plant sciences such as energy, water and nutrient use efficiency, and to future societal challenges.

Required qualifications include an earned doctorate in biology or related field at time of application, a record of research accomplishment in plant physiology, record of ability to teach undergraduate and graduate courses in plant biology, including physiology, effective communication skills, and demonstrated ability to collaborate with other scientists. Duties include developing and maintaining an active research program supported by extramural funding, training graduate and undergraduate students, teaching graduate and undergraduate courses in biology, participating in service needs, and advancing our commitment to diversity and multiculturalism.

To apply, visit www.wsujobs.com to upload application materials. Applications must include a letter of application addressing qualifications, a curriculum vitae, separate teaching and research statements and up to five selected reprints of published or in press papers. Three (3) letters of recommendation that address the applicant's history of and potential for research, teaching and communication excellence are required. The reference letters will be automatically requested and obtained from the reference provider through our online application system. Review of applications begins **November 12, 2013**. For information on the position or the status of your application, candidates may contact **Dr. Mechthild Tegeder** (tegeder@wsu.edu). Full notice of vacancy can be viewed at <https://www.wsujobs.com>.

EEO/AA/AD



California State Polytechnic University, Pomona Biological Sciences Department

TENURE-TRACK FACULTY POSITION PLANT GENETICIST

The Biological Sciences Department at California State Polytechnic University, Pomona (Cal Poly Pomona) invites applications for a tenure-track, ASSISTANT PROFESSOR position in Plant Genetics, beginning September 2014. The area of specialty is open, but candidates who use molecular or computational tools to understand aspects of plant development, stress response, epigenetics, genomics, evolution and/or ecology are encouraged to apply. A Ph.D. in biology, botany, genetics, or a related field is required. Post-doctoral experience and previous college teaching experience are preferred. The successful candidate will have the potential for excellence in undergraduate teaching, and for developing an externally-funded research program that will involve undergraduate and Master's students. Teaching responsibilities will include plant and genetics courses, specialty courses in the candidate's area of expertise, and may involve participation in introductory biology and other courses in botany. Cal Poly Pomona is a comprehensive Master's university with a diverse student body. The successful candidate will have demonstrated an ability to be responsive to the educational equity goals of the university and its increasing ethnic diversity and international character.

Applicants should forward: (1) a cover letter that briefly describes the candidate's training, experience, and teaching and research interests; (2) curriculum vitae; (3) statement of teaching philosophy; (4) proposed plan of research; (5) representative publication reprints; and (6) the names and contact information of five references to: **Chair, Plant Genetics Search Committee, Biological Sciences Department, California State Polytechnic University, 3801 West Temple Avenue, Pomona, CA 91768**. Electronic submission of application materials as a single PDF file is preferred (plantgene_search@csupomona.edu). Review of applications begins on **December 2, 2013**. Official transcripts and three letters of reference will be required of all finalists. For further information, visit the Department web site at: <http://www.csupomona.edu/~biology>.

California State Polytechnic University, Pomona is an Equal Opportunity, Affirmative Action Employer.

Faculty Position in Sensory Neurobiology Department of Biological Sciences Purdue University

Purdue University is recruiting 6 faculty members who will enhance ongoing efforts in autism research. Areas of specialization are expected to range from the molecular, cellular and organismal to the behavioral, systems and educational levels. Current searches associated with the Autism Cluster are in Biological Sciences, Education, Psychological Sciences and Speech, Language and Hearing Sciences, <http://www.purdue.edu/hhs/psy/autismclusterhires.php>.

The Dept of Biological Sciences invites applicants for a tenure-track Assistant Professor position in the area of Sensory Neurobiology. We seek candidates whose research focuses on the neurobiology underlying any of the major senses at the molecular, cellular or organismal levels. Approaches of interest include circuit and optogenetics, electrophysiology, advanced imaging, and animal models of disease, particularly those with implications for autism. Applicants must have a Ph.D. or equivalent in neurobiology or a related discipline and at least 2 years of postdoctoral experience. The successful applicant will conduct research, teach undergraduate and graduate students, and participate in ongoing programs in the Department.

The Department has over 50 faculty members conducting research in diverse fields including neurobiology, cell/molecular/developmental biology, behavior, evolution, ecology, microbiology/virology, structural biology, and bioinformatics. For further information about the Department visit <http://www.bio.purdue.edu/>. The successful candidate will have opportunities to interact with neurobiologists and allied scientists across the University, including the Purdue University Interdisciplinary Neuroscience Program and the Purdue Autism Cluster. First-rate laboratory facilities are available in Lilly Hall, allied Centers, and new facilities in the Jischke Hall of Biomedical Engineering and the Hockmeyer Hall of Structural Biology.

Applications and inquiries must be submitted electronically to <https://hiring.science.purdue.edu>. Applications must be in the form of a PDF file that includes a detailed curriculum vitae, names and addresses of three referees, a 2 - 3 page summary of research interests, and a one-page teaching statement. Search@bio.purdue.edu. Review of applications will begin **December 1, 2013** and continue until the positions are filled. A background check will be required for employment in this position.

Purdue University in an Equal Opportunity/Equal Access/Affirmative Action Employer fully committed to achieving a diverse workforce.

FACULTY CLUSTER HIRE APPOINTMENTS

Microbial Systems for the Bioremediation of Water and Soils

ARE YOU A CREATIVE INVESTIGATOR WHO EXCELS AT WORKING COLLABORATIVELY AND PUSHING DISCIPLINARY BOUNDARIES?

The University of Minnesota announces hiring for an interdisciplinary research cluster focused on the use of bioremediation to conserve the environment and advance industry. The first in a hiring series supported by Minnesota Discovery, Research and InnoVation for Minnesota's Economy (MnDRIVE), this initiative focuses on using scientific discovery and innovation to enhance efficient environmental stewardship and to position the state as a leader in key industries.

The University, in the first phase, will fill four faculty positions in the Departments of Civil Engineering; Soil, Water, and Climate; and Biochemistry, Molecular Biology and Biophysics. In particular, new faculty hires will be sought with expertise in metal transformations, the degradation and remediation of organic contaminants, and the recovery and treatment of inorganic nutrients.

All faculty members will have co-appointments in the Biotechnology Institute (BTI) to facilitate interactions between faculty across multiple colleges and departments and with Minnesota industries.

All positions are tenure track, with 9 month appointments. Successful candidates will have research expertise that complements current faculty and be committed to graduate and undergraduate education. The University of Minnesota is an equal opportunity educator and employer.



UNIVERSITY
OF MINNESOTA
Driven to Discover™

LEARN MORE AT

z.umn.edu/bioremediationhiring

UNIVERSITY OF MINNESOTA



Florida State University Faculty Recruitment for an Interdisciplinary Initiative in Brain Health and Disease

Florida State University is pleased to announce a major interdisciplinary faculty hiring initiative in the broadly defined area of **mechanistic and translational approaches to brain health and disease**. As many as nine tenure-track/tenured faculty positions will be filled. This faculty search is open with respect to rank and academic department. We invite nominations and applications from researchers conducting basic science (including research on nonhuman animals), clinical/translational science, cognitive, behavioral, social and developmental neuroscience as well as computational, bio-informatic, specialized imaging, and genetic approaches to studying brain health and disease. Areas of interest include, but are not limited to, brain systems that regulate normal and abnormal behavioral and cognitive functions across the life span, brain mechanisms underlying psychiatric, neurodevelopmental, and neurodegenerative disorders, and the effects of environmental and social influences on brain function.

Successful candidates are expected to be using innovative methodologies and approaches to the study of brain function and to have a synergistic impact on existing research programs in the University's departments and interdisciplinary centers (http://www.research.fsu.edu/brain-initiative_search/) as well as open up new areas. Sustained pursuit of collaborative, externally-funded projects is an explicit goal to be addressed by this initiative. Successful candidates will be offered highly competitive salaries and start-up packages, state-of-the-art research space and access to world-class instrumentation and facilities in academic and interdisciplinary units.

Florida State University is a Carnegie RU/VH (very high research activity) institution with a student population exceeding 41,000. The University is located in Tallahassee, the Capital of Florida, where residents have access to a broad range of cultural amenities afforded by the presence of three institutions of higher learning. The region is in close proximity to the Apalachicola National Forest and boasts an abundance of springs, lakes and rivers as well as pristine beaches and the adjacent waters of the Gulf of Mexico.

Applicants are asked to provide a single document in PDF format containing a letter of application, a full CV, the names and contact information of three references, and a two page narrative describing their research interests that should include links to recent publications and a clear statement as to how the candidate would complement this inter-college hiring initiative at Florida State University. Send full applications to brain-initiative.search@fsu.edu. For full consideration, applications should be received by **November 15, 2013**.

*Florida State University is committed to the diversity of its faculty, staff, and students, and to sustaining a work and learning environment that is inclusive. Women, minorities, and people with disabilities are encouraged to apply.
FSU is an Equal Opportunity/Access/Affirmative Action Employer.*

Careers in Neuroscience

Special Career Feature: November 1, 2013

Reserve your ad by October 15 to guarantee space.*

*Ads accepted until October 28 if space is still available.

Ask about customized packages for

*Ads accepted until October 28 if space is still available.

Ask about customized packages for this feature

Produced by the *Science*/AAAS Custom Publishing Office

Are you a creative biomedical scientist that pushes disciplinary boundaries and embraces collaborative work?

Then we want to hear from you.

The Department of Integrative Biology & Physiology—a new department in the University of Minnesota Medical School—is hiring tenure-track faculty at all ranks. Our mission involves gene to whole organism, focusing on cardiovascular, muscle, obesity, diabetes and metabolism.

Successful candidates will have expertise that complements current faculty and be committed to graduate and undergraduate education.

Learn more at
<http://physiology.med.umn.edu>

*The University of Minnesota is an Equal Opportunity
Educator and Employer.*

Keck School of Medicine of USC

Department of Stem Cell Biology and Regenerative Medicine

Assistant Professorships in Stem Cells and Regenerative Medicine and Stem Cells Tissue Engineering

The Department of Stem Cell Biology and Regenerative Medicine is recruiting candidates whose research focuses on understanding fundamental principles of regenerative processes and developing knowledge-based approaches to organ repair. The Department is housed within the newly created Eli and Edythe Broad CIRM Center for Regenerative Medicine and Stem Cell Research within the Keck School of Medicine at the University of Southern California. Significant resources are available to support all aspects of stem cell research within the building and adjacent centers. Excellent collaborative opportunities exist across the USC campuses. The Department is seeking scholars researching and translating regenerative mechanisms of tissue repair. More specifically, the Department is interested in applicants employing tissue engineering to regenerative medicine. In addition to its research mission, all members will play an important role in the educational mission of this newly created Department. Generous start-up packages will be awarded to the successful candidates.

Online applications will be accepted for each search, please apply to <https://jobs.usc.edu/applicants/Central?quickFind=72396> for both the search in Stems Cells and Regenerative Medicine and Stem Cells Tissue Engineering. Applications should include a letter of interest, curriculum vitae, brief 2-3 page outline of research past, present and future, and four letters of reference. The applicant is responsible for ensuring the completed application is received before **November 21st, 2013**.

*Women and individuals belonging to minority groups are particularly encouraged to apply. The University of Southern California is an
Equal Opportunity Affirmative Action Employer.*

“More than 20 of the brightest minds on the planet.” BBC



FALLING WALLS CONFERENCE 2013 BERLIN 8/9 NOV

WHICH ARE
THE NEXT
WALLS
TO FALL?



FALLING WALLS is a unique international platform for leaders from the worlds of science, business, politics, the arts and society.

minutes each the breakthroughs they are working on.

THE FALLING WALLS CONFERENCE (9 November, 9:00–19:00) is at the heart of the Falling Walls days in Berlin. Over 20 researchers from top institutions around the globe present in only 15

The **Falling Walls Venture** (8 November, 13:00–18:00) is an international forum for outstanding science based start-up companies, venture capital companies and strategic investors. It is supported by the European Private Equity and Venture Capital Association (EVCA).

REGISTER BEFORE 30 OCTOBER 2013
www.falling-walls.com/registration

More information
and registration:
www.falling-walls.com

Science Careers is the forum that answers questions.



Science Careers is dedicated to opening new doors and answering questions on career topics that matter to you. With timely feedback and a community atmosphere, our careers forum allows you to connect with colleagues and experts to get the advice and guidance you seek as you pursue your career goals.

Science Careers Forum:

- » Relevant Career Topics
- » Timely Advice and Answers
- » Community, Connections, and More!

Visit the forum and join the conversation today!



Your Future Awaits.



ScienceCareers.org

Assistant or Associate Professor



Perelman
School of Medicine
UNIVERSITY of PENNSYLVANIA

The Department of Cancer Biology at the Perelman School of Medicine at the University of Pennsylvania seeks candidates for an Assistant, Associate, and/or Full Professor position in the tenure track. Rank will be commensurate with experience. The successful applicant will have experience in the field of cancer biology, including but not limited to cancer genetics and genomics, tumor microenvironment, chromosome biology, cancer cell metabolism and oncogenic signaling.

Responsibilities include the development of an independent research program. Teaching duties comprise mentoring students and course lecturing. Applicants must have an M.D. and/or Ph.D degree and have demonstrated excellent qualifications in education and research.

We seek candidates who embrace and reflect diversity in the broadest sense. The University of Pennsylvania is an equal opportunity, affirmative action employer.

Apply for this position online at: http://www.med.upenn.edu/apps/faculty_ad/index.php/g304/d3418

UT SOUTHWESTERN MEDICAL CENTER

The University of Texas Southwestern Medical Center is accepting applications for an experienced specialist to assist in mouse embryo transfer, transgenesis, ICSI, and stem cell work in the rapidly expanding Center for the Genetics of Host Defense under the direction of Dr. Bruce Beutler.

Competitive applicants must have a Bachelor's or Master's degree with existing proficiency with these techniques preferred. Teamwork is essential. Please send CV including contact information to:

Bruce Beutler, M.D.

**Regental Professor and Director
Center for Genetics of Host Defense
Raymond and Ellen Willie
Distinguished Chair in Cancer Research
in honor of**

**Laverne and Raymond Willie Sr.
The University of Texas
Southwestern Medical Center at Dallas
5323 Harry Hines Boulevard
Dallas, Texas 75390-8505
Telephone: (214) 648-5838**

Email:

**Bruce.Beutler@utsouthwestern.edu
Visit Mutagenetix:
<http://mutagenetix.utsouthwestern.edu>**

*UT Southwestern is an Affirmative Action/
Equal Opportunity Employer. Women,
minorities, veterans, and individuals with
disabilities are encouraged to apply.*



FACULTY POSITION IN SOFT MATERIALS AT DREXEL UNIVERSITY

The Department of Materials Science & Engineering at Drexel University (www.mse.drexel.edu) is seeking applications for a tenured/tenure-track faculty position with a demonstrated record of excellence in original research in soft materials. While primary consideration will be given to candidates with areas of expertise in synthesis and processing of polymeric materials, the ideal applicant should possess research interests in applying principles of soft materials in emerging research areas such as advanced energy technologies, biomedical materials and devices, or environmental sustainability. Located in an exciting urban environment, our department has rapidly expanded during the past 10 years; presently has 16 faculty, 155 undergraduate and over 100 graduate students working in four core research directions including materials for energy, health, extreme environments and electronics. Our graduate program was recently ranked #11 among all materials PhD programs in the US by the National Research Council. To apply, please go to our application page <http://www.materials.drexel.edu/faculty/positions/>. The position is available immediately and applications will be considered until the position is filled.

Drexel University is an Equal Opportunity Employer and encourages applications from qualified women and minorities.

There's only one

DR. SHIRLEY MALCOM



To Dr. Shirley Malcom, born and raised in the segregated South more than 65 years ago, a career based on her studies in science seemed even less likely than the launch of the Soviet's Sputnik. But with Sputnik's success, the Space Race officially started and, in an instant, brought a laser-like focus to science education and ways to deliver a proper response. Not long after, Dr. Malcom entered the picture.

Although black schools at the time received fewer dollars per student and did not have sufficient resources to maintain their labs at a level equivalent to the white schools, Dr. Malcom found her way to the University of Washington where she succeeded in obtaining a B.S. in spite of the difficulties of being an African American woman in the field of science. From there she went on to earn a Ph.D. in ecology from Penn State and held a faculty position at the University of North Carolina, Wilmington.

Dr. Malcom has served at the AAAS in multiple capacities, and is presently Head of the Directorate for Education and Human Resources Programs. Nominated by President Clinton to the National Science Board, she also held a position on his Committee of Advisors on Science and Technology. She is currently a member of the Caltech Board of Trustees, a Regent of Morgan State University, and co-chair of the Gender Advisory Board of the UN Commission on Science and Technology for Development. She has held numerous other positions of distinction and is the principal author of *The Double Bind: The Price of Being a Minority Woman in Science*.

Of her active career in science, Dr. Malcom says, "I guess I have become a poster child for taking one's science background and using that in many other ways: we ask questions; we try to understand what we find; we consider what evidence we would need to confirm or refute hypotheses. And that happens in whatever setting one finds oneself."

At *Science* we are here to help you in your own scientific career with expert career advice, forums, job postings, and more — all for free. Visit *Science* today at ScienceCareers.org.

**AAAS**

For your career in science, there's only one **Science**

ScienceCareers.org



ASSISTANT PROFESSOR OF PHYSIOLOGY

The Department of Physiology at the Perelman School of Medicine at the University of Pennsylvania seeks candidates for an Assistant Professor position in the tenure track. Responsibilities include establishing and conducting an independent research program, and supervising, mentoring, and teaching students. Applicants must have an M.D., Ph.D., or equivalent degree and have demonstrated excellent qualifications in research.

The successful applicant will have experience in any aspect of physiology, ranging from integrative to cell biological to molecular biophysical. Emphasis will be placed on novelty and impact of the research, with special consideration given to candidates employing cutting-edge approaches. Please submit a cover letter, curriculum vitae, the names and contact information of three references, and a two- to four-page statement of your research interests.

First consideration will be given to applications received by November 30, 2013, but applications will continue to be accepted after this date.

We seek candidates who embrace and reflect diversity in the broadest sense. The University of Pennsylvania is an Equal Opportunity/Affirmative Action Employer.

Apply for this position online only at **website:** http://www.med.upenn.edu/apps/faculty_ad/index.php/g311/d3426.

ASSISTANT PROFESSOR—ALL AREAS Princeton University Department of Chemistry

The Department of Chemistry at Princeton University invites applications for a tenure-track assistant professor position in all areas of chemistry. We seek faculty members who will create a climate that embraces excellence and diversity, with a strong commitment to teaching and mentoring that will enhance the work of the department and attract and retain students of all races, nationalities, and genders. We strongly encourage applications from members of all underrepresented groups. Candidates are expected to have completed the Ph.D. in chemistry or a related field at the time of appointment. Applicants should submit a description of research interests, curriculum vitae, a list of publications, and contact information for three references online at **website:** <http://jobs.princeton.edu/applicants/Central?quickFind=64207>. The search committee will begin review of applications on October 17, 2013 and will continue until the position is filled.

Princeton University is an Equal Opportunity Employer and complies with applicable EEO and Affirmative Action regulations.

FACULTY POSITION PRIVATE University of Wisconsin-Madison

The Department of Comparative Biosciences, School of Veterinary Medicine invites applications for tenure-track faculty position (ASSISTANT/ASSOCIATE PROFESSOR). Qualifications include Ph.D. or equivalent, postdoctoral experience, ability to develop extramurally funded research program and commitment to excellent teaching. Research area is open, but preference given to neuroplasticity, mammalian epigenetics and/or host/microbiome interactions. Teaching responsibilities based on expertise. Send cover letter, curriculum vitae, brief statements of research interests and goals and teaching philosophies and experience, and three letters of reference to: **Hannah Carey, Professor, Department of Comparative Biosciences, University of Wisconsin, 2015 Linden Dr., Madison, WI, 53706.** Apply by December 15, 2013. Send application to e-mail: cbs_admin@vetmed.wisc.edu. For additional information, see **website:** <http://www.vetmed.wisc.edu/about-the-school/employment/>. Equal Opportunity/Affirmative Action Employer.



2014 POSTDOCTORAL RESEARCH FELLOWSHIPS

In support of its 2020 Vision & Strategic Plan, Mote Marine Laboratory will award fellowships in 2014 for three tracks: any marine research field; general coastal ecology, and shellfish/benthic ecology. Two fellowships are expected to begin between January and August 31 with a third fellowship expected to begin by December 31. A Ph.D. must have been awarded by start of fellowship award period and only applicants who received the Ph.D. (or equivalent) on or after December 2010 will be considered. For complete Fellowship information and application requirements see **website:** <http://www.mote.org/postdocs>.

Mote Marine Laboratory is an Equal Opportunity Employer/ADA/E-Verify Employer.

TENURE-TRACK FACULTY POSITION Forensic Biology

The Department of Biology (**website:** <http://biology.iupui.edu>) and the Forensic and Investigative Science Program (**website:** <http://forensic.iupui.edu>) in the School of Science at Indiana University-Purdue University Indianapolis (IUPUI) invite applications for a tenure-track faculty position in Forensic Biology to begin August 1, 2014. Applicants with interests in genetics, molecular biology, bioinformatics, or a related area applicable to forensic science are encouraged to apply. Applicants must have a background appropriate for successful teaching in interdisciplinary B.S. and M.S. degree programs in Forensic and Investigative Sciences. A Ph.D. with postdoctoral experience is required, together with demonstrated evidence of research excellence and the ability to initiate and maintain an externally funded research program. The Forensic and Investigative Sciences Program has an eight-year-old, accredited B.S. degree with more than 120 majors and a M.S. degree. Both the B.S. and M.S. degrees have concentrations in forensic biology and forensic chemistry. One PDF file should be submitted to e-mail: forensicscience@chem.iupui.edu that includes a cover letter, curriculum vitae, and statement of research plans (max five pages) and teaching interests/experience (max two pages). Candidates should arrange to have three letters of recommendation sent to the same e-mail address. Evaluation of completed applications will begin November 1, 2013 and will continue until the position is filled.

IUPUI is an Equal Employment Opportunity/Affirmative Action Employer, Minorities/Females/Persons with Disabilities.

MARINE AND COASTAL SCIENCES

The Department of Ecology and Evolutionary Biology at Tulane University seeks a full-time, non-tenure-track PROFESSOR OF THE PRACTICE (PoP) beginning fall 2014. The PoP will administer marine biology minors and teach introductory marine biology and other courses in marine and coastal sciences. An earned doctorate in biological sciences or other appropriate field is required. We seek an exceptional individual with a commitment to excellence in undergraduate education. PoPs are appointed for initial three-year, renewable terms. More information about the position can be found at **website:** <http://tulane.edu/sse/eebio/about/pop.cfm>. To apply, submit curriculum vitae, statement of teaching philosophy and proposed classes, description of scholarly and teaching interests and experience, and the names and addresses of three references electronically to e-mail: ecolevol@tulane.edu. Review of applications will begin November 15, 2013, and the position will remain open until filled. *Tulane University is an Affirmative Action/Equal Employment Opportunity/ADA Employer committed to excellence through diversity. All eligible candidates are encouraged to apply. This position is subject to final budgetary approval.*

U.S. POSTAL SERVICE

Statement required by the Act of 12 August 1970, Section 3685, Title 39, United States Code, showing the ownership, management, and circulation of:

1–9. *Science*, Publication No. 0036-8075, is published weekly on Friday, except the last week in December, at 1200 New York Avenue, N.W., Washington, DC 20005. Date of filing: 25 September 2013. This is also the address of the publisher, the editor, and the managing editor, who are, respectively, Beth Rosner, Marcia McNutt, and Monica M. Bradford.

10. The owner is the American Association for the Advancement of Science, 1200 New York Avenue, N.W., Washington, DC 20005. Stockholders: None.

11. Known bondholders, mortgages, and other security holders owning or holding 1 percent or more of total amount of bonds, mortgages, or other securities: None.

12. The purpose, function, and nonprofit status of this organization and the exempt status for federal income tax purposes have not changed during the preceding 12 months.

13–15. The average number of copies of each issue during the preceding 12 months is (A) Total number of copies printed: 109,810; (B) Paid circulation: 88,310; (1) Paid/Requested outside-county mail subscriptions stated on form 3541: 74,343; (2) Paid/Requested in-county subscriptions stated on form 3541: 0; (3) Sales through dealers and carriers, street vendors, counter sales: 13,955; (4) Other classes mailed through USPS: 12; (C) Total paid circulation: 88,310; (D) Free distribution: samples, complimentary, and other free copies: 19,494; (1) Outside-county as stated on form 3541: 2,395; (2) In-county as stated on form 3541: 0; (3) Other classes mailed through the USPS: 2; (4) Free distribution outside of mail carrier or other means: 17,097; (E) Total free distribution: 19,494; (F) Total distribution: 107,804; (G) Copies not distributed: 2,006; (H) Total: 109,810; (I) Percent paid and/or Requested Circulation: 81.9%.

Actual number of copies of single issue (9/13/2013) published nearest to filing date are (A) Total number of copies printed: 91,300; (B) Paid circulation: 85,546; (1) Paid/Requested outside-county mail subscriptions stated on form 3541: 72,458; (2) Paid/Requested in-county subscriptions stated on form 3541: 0; (3) Sales through dealers and carriers, street vendors, counter sales: 13,078; (4) Other classes mailed through USPS: 10; (C) Total paid circulation: 85,546; (D) Free distribution: Samples, complimentary, and other free copies: 3,720; (1) Outside-county as stated on form 3541: 2,446; (2) In-county as stated on form 3541: 0; (3) Other classes mailed through the USPS: 2; (4) Free distribution outside of mail: Carrier or other means: 1,272; (E) Total free distribution: 3,720; (F) Total distribution: 89,266; (G) Copies not distributed: 2,034; (H) Total: 91,300; (I) Percent paid and/or Requested Circulation: 95.8%.

I certify that the statements made above are correct and complete. (signed) Beth Rosner, Publisher.

Help employers find
you. Post your
resume/cv.

Science Careers

From the journal *Science*



www.ScienceCareers.org

Get your questions answered.
Careers Forum
www.ScienceCareers.org

our CWC data set (21), suggesting additional paternal genetic links to Kurgan cultures. Together with the affinities of the CWC to present-day populations of Eastern Europe, the Baltics, and the Caucasus (figs. S4G to S7G), this suggests a genetic influx into Central Europe from the East, likely influenced by Kurgan cultures (movie S1) (2, 3).

Event D (~2500 cal BCE) is defined by the BBC (movie S1), the western counterpart of the CWC (fig. S2) (2–4). BBC groups appeared ~300 years later in Mittelbe-Saale and coexisted alongside the CWC for more than 300 years (4). The BBC is distinguished from the CWC by the absence of haplogroup I and U2 and an overwhelmingly dominant genetic signature of haplogroup H (48.3%) (fig. S3), leading to a separation of the BBC from all other Mittelbe-Saale cultures in PCA and cluster analysis (Fig. 1, C and D). H remains the most frequent haplogroup in West European populations today (~40%) (8, 9) and was absent in Central European hunter-gatherers (10, 14) but prevalent in ancient populations of the Iberian Peninsula since Mesolithic times (20.7 to 70.7%) (table S9) (22–24). As a result, the BBC clusters with these Iberian populations (Fig. 1, C and D), whereas the results from Procrustes and MDS were less informative. However, genetic links between the BBC and modern Iberian populations were supported by genetic distance maps accounting for H sub-haplogroups (fig. S7H) and ancient mitochondrial H genomes (12). These suggest the BBC was associated with a genetic influx from southwest Europe (movie S1), which is consistent with the oldest archaeological signs of this culture being found in Portugal ~2800 cal BCE (2, 3).

The onset of the EBA in Mittelbe-Saale (~2200 cal BCE) was characterized by socially and economically stratified societies associated with the emerging metallurgies (2–4). All of the analyses show close genetic links between the EBA and the CWC (Figs. 1, C and D, and 2A) on the basis of elevated frequencies of Late Neolithic/EBA haplogroups such as I, U2, and T1 (22.3%) (Figs. 1C and 3 and fig. S3), and both appear to have similar affinities to modern-day East European populations (movie S1 and figs. S5I to S8I). TPC (Fig. 2D) indicate a minimal contribution of the BBC to the EBA in Central Europe. Thus, the Late Neolithic/EBA in Mittelbe-Saale appears to have witnessed rapid and dynamic changes in mtDNA composition at the crossroads of distinct Eastern and Western European influences (movie S1).

To investigate the potential impact of the geographically widespread archaeological cultures and events examined here (fig. S2) on the demography and genetic variation of present-day

Central Europeans, we compared the ancient data with a Central European metapopulation (CEM) consisting of 500 randomly selected individuals (13). AMOVA supports a model of continuity from the Late Neolithic/EBA to the CEM with the best inter- and intragroup variance observed when all Late Neolithic/EBA samples are pooled with the CEM into one group and the Early/Middle Neolithic specimens into another (among groups 2.57% , $F_{st} = 0.02572$, $P = 0.00891$; within groups 0.50% , $F_{st} = 0.00511$, $P = 0.08089$) (table S14). TPC analyses also support continuity since the Late Neolithic/EBA ($P = 0.134672$ to 0.418949) (Fig. 2D). Similarly, Bayesian coalescent-based simulations (13) support a demographic model involving exponential population growth since the Neolithic with a contribution of at least 50% migrants to Mittelbe-Saale during the Early Neolithic. This is followed by a constant ratio of gene flow/admixture between Early/Middle and incoming Late Neolithic/EBA components and, after this fusion, a genetic continuity until the present day (Akaike Information Criterion 99.9%) (fig. S8 and table S15). The fact that continuity since the Late Neolithic/EBA could not be rejected confirms that the succeeding events B to D, despite their differing geographic affinities, had formed today's mtDNA diversity. Notably, the CEM clusters with the Late Neolithic cultures and individuals of the BBC in particular (Fig. 2A), suggesting that the Western European mtDNA variability had a stronger influence than the contemporaneous eastern CWC/EBA complex, implying yet another shift after the EBA.

We evaluated the amount of lineages in the CEM that can be attributed to particular time periods by characteristic haplogroups (13) and found that a total of 53% can currently be assigned to the Palaeolithic/Mesolithic (16%), Early/Middle Neolithic (31.2%), and Late Neolithic periods (5.8%) (Fig. 3). The remaining proportion of lineages (47%, mainly haplogroup H) requires further resolution (12). The presence of all major mtDNA haplogroups by the end of the Neolithic makes it increasingly difficult to discern recent demographic changes and would require larger population events to have an observable effect and/or full mitochondrial genome sequencing to detect more subtle changes.

The detailed genetic analyses of this transect through Neolithic Central Europe demonstrate the key role of Late Neolithic cultures at the dawn of metallurgy and stratified societies in the formation of modern Central European mtDNA diversity. The four successive genetic shifts highlight the biological cohesiveness of archaeological cultures such as the LBK, FBC, CWC, and BBC cultures and the importance and dynamics of genetic input from different geographic regions.

References and Notes

1. A. W. R. Whittle, V. Cummings, Eds., *Going Over: The Mesolithic-Neolithic Transition in North-West Europe* (Oxford Univ. Press, Oxford, 2007).
2. P. Bogucki, P. J. Crabtree, Eds., *Ancient Europe 8000 B.C.-A.D. 1000. Encyclopaedia of the Barbarian World* (Scribner, New York, 2004).
3. B. Cunliffe, Ed., *Europe Between the Oceans: Themes and Variations: 9000 BC–AD 1000* (Yale Univ. Press, New Haven, CT, 2008).
4. H. Behrens, *Die Jungsteinzeit im Mittelbe-Saale-Gebiet* (Veröffentlichungen des Landesmuseums für Vorgeschichte in Halle 27, Berlin, 1973).
5. R. Pinhasi, M. G. Thomas, M. Hofreiter, M. Currat, J. Burger, *Trends Genet.* **28**, 496–505 (2012).
6. P. Soares et al., *Curr. Biol.* **20**, R174–R183 (2010).
7. A. J. Ammerman, L. L. Cavalli-Sforza, *The Neolithic Transition and the Genetics of Populations in Europe* (Princeton Univ. Press, Princeton, NJ, 1984).
8. M. Richards et al., *Am. J. Hum. Genet.* **67**, 1251–1276 (2000).
9. A. Achilli et al., *Am. J. Hum. Genet.* **75**, 910–918 (2004).
10. B. Bramanti et al., *Science* **326**, 137–140 (2009); 10.1126/science.1176869.
11. W. Haak et al., *PLOS Biol.* **8**, e1000536 (2010).
12. P. Brotherton et al., *Nat. Commun.* **4**, 1764 (2013).
13. Materials and methods are available as supplementary materials on Science Online.
14. Q. Fu et al., *Curr. Biol.* **23**, 553–559 (2013).
15. H. Malmström et al., *Curr. Biol.* **19**, 1758–1762 (2009).
16. P. Skoglund et al., *Science* **336**, 466–469 (2012).
17. C. Der Sarkissian et al., *PLOS Genet.* **9**, e1003296 (2013).
18. J. Krause et al., *Curr. Biol.* **20**, 231–236 (2010).
19. C. Keyser et al., *Hum. Genet.* **126**, 395–410 (2009).
20. C. Lalueza-Fox et al., *Proc. Biol. Sci.* **271**, 941–947 (2004).
21. W. Haak et al., *Proc. Natl. Acad. Sci. U.S.A.* **105**, 18226–18231 (2008).
22. M. Hervella et al., *PLOS ONE* **7**, e34417 (2012).
23. C. Gamba et al., *Mol. Ecol.* **21**, 45–56 (2012).
24. M. Lacan et al., *Proc. Natl. Acad. Sci. U.S.A.* **108**, 9788–9791 (2011).

Acknowledgments: Sequence data have been deposited in GenBank (www.ncbi.nlm.nih.gov/genbank) under the accession numbers KF600801 to KF601193. The skeletal remains investigated in this study are archived in the State Museum of Prehistory of Saxony-Anhalt, Halle (Saale), Germany. We thank R. Schwarz, L. Weyrich, C. Knipper, J. Tuke, N. Patterson, I. Lazaridis, and E. Bánffy for reading and critical discussion of the Manuscript; O. Balanovsky for providing population data from Russia, Ukraine, and Belarus; C. Metzner-Nebelsick and V. Hubensack for archaeological information about the Leau, Röcken, and Plötzkau sites; J. Osthof and G. Krizma for informatics support; and B. Bramanti for investigations of the Benzingerode site. This research was supported by the German Research Foundation, the Geocycles Earth System Research Center at the University of Mainz, and the Genographic Project. The Genographic Project is supported by funding from the National Geographic Society, IBM, and the Waitt Family Foundation.

Supplementary Materials

www.sciencemag.org/content/342/6155/257/suppl/DC1

Materials and Methods

Supplementary Text

Figs. S1 to S10

Tables S1 to S17

References (25–91)

Movie S1

12 June 2013; accepted 12 September 2013

10.1126/science.1241844